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A COMPARATIVE ANALYSIS OF MHC GENETIC DIVERSITY AT THE CLASS II LOCI IN SOME ARCTIC MAMMALIAN SPECIES

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirement

for the Degree of

DOCTOR OF PHILOSOPHY

By

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Fairbanks, Alaska

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A comparative analysis of MHC genetic diversity at the class II loci in some arctic mammalian species

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Abstract

The genetic diversity at the Major Histocompatibility Complex (MHC) class II loci in some arctic mammalian species, musk ox, moose, caribou, and bears, have been characterized. The general objective of this study was to broaden the knowledge of the MHC polymorphism, selection, evolution and function in natural populations of arctic mammals. Allelic variation was assessed by analysis of MHC class II DR and DQ loci at exon 2 region. Sequences were amplified via the polymerase chain reaction (PCR), followed by either DNA sequencing after cloning of the PCR products or single-stranded conformation polymorphism (SSCP) analysis and sequencing.

Monomorphism was observed at DRA, DRB, and DQA loci in both musk ox and moose, but relatively high polymorphism was observed at DQB locus. For the first time, four DQB alleles and one DQA allele were found identical in these two distantly related species which split approximately 23 million years ago, indicating stringent trans-species polymorphism. Both DRB and DQB seem to be functional by analyzing their cDNA expression.

An intermediate level of MHC polymorphism at DRB locus was found in caribou and reindeer. Phylogenetic analysis of cervid DRB alleles indicated that all reindeer and caribou DRB alleles were from a monophyletic lineage, implying an ancient bottleneck in *R. tarandus*.

High polymorphism at the DRB locus in polar bear was also observed. Four DRB alleles were found to be shared by polar bear and dog. The trans-species polymorphism of the shared alleles may have been persistent for 10 to 15 million years.

Nine DQB alleles rather than two DRB alleles were also found in a pure domestic dog lineage of Doberman pinschers. These data imply that selection pressure may vary among MHC loci.

In summary, the general level of MHC polymorphism at the class II loci is lower in herbivores (musk ox, moose, and caribou) than carnivores (polar bear). Biased selection may be applied on DQB locus. Stringent trans-species polymorphism between two distantly related species may be the result of persistent selection by shared parasites in the same environment.

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Chapter I

Literature review

Introduction:

The major histocompatibility complex (MHC) has persisted in vertebrates as a multigene family for about 450 million years. It descended from jawed fishes to amphibians, reptiles, birds, and mammals (Klein and Horejsi 1997). The MHC was discovered in 1936 by Gorer who inferred its role when the mouse tumors he transplanted were rejected (Klein 1986). Zinkernagel (1974) and Doherty (1975) suggested that the function and polymorphism of MHC molecules is to bind and present antigens (peptides) to the T cells. This suggestion has been confirmed by x-ray crystallographic structure analyses (Bjorkman et al. 1987 and Brown et al. 1988 & 1993).

For more than a half century, the MHC has been one of the most thoroughly studied gene complexes, especially in humans, with over a hundred MHC alleles found at some loci and the whole MHC genome sequenced (The MHC sequencing consortium 1999). It is now understood that not only will foreign tissue initiate graft rejection, but that a vast variety of different pathogens and parasites can also initiate immune responses. It is generally believed that the wide spectrum of pathogens and parasites is the major driving force that accounts for the MHC polymorphism (Doherty and Zinkernagel 1975, Klein and O'hUigin 1994).

The MHC is a physically linked large gene complex that contains many different loci (Trowsdale 1996). The majority of genes from these loci are more or less involved in the presentation of antigenic peptides to T cells which subsequently activate the immune system (Klein 1986). In humans, the MHC is located on the short arm of chromosome 6 (6p21.3) and is called human leukocyte antigen (HLA). It is 4 megabases (4000 kb) in overall length and contains 224 genes (Trowsdale 1996 and The MHC sequencing consortium 1999). In contrast, the MHC region in chickens is much reduced in size, only 92 kb in length containing 19 genes (Kaufman et al. 1999).

HLA as well as MHC in general in all vertebrates comprises two major regions: class I and class II. Products of both these classes are involved in presenting endogenous antigens (from intracellular sources, such as virus coat proteins or self peptides, for example) and exogenous (taken up from extracellular sources, such as bacterial and self peptides) antigens. A third region called class III usually separates class I and class II (except chicken in which class III is on the outside, flanking the class I region; Kaufman et al. 1999) and contains unrelated genes (Fig. 1.1). Some of the class I and class II genes have extraordinarily high levels of variability, among the highest known in any organism studied thus far (Klein 1986). In addition, several lines of evidence have demonstrated that the polymorphism at certain MHC loci is selectively maintained rather than selectively neutral, which makes these MHC loci unusual (Hughes and Neil 1988, 1989). Furthermore, the polymorphism of MHC genes has a strong association, conferring either resistance or susceptibility, with various kinds of diseases, including HIV, malaria, hepatitis, and diabetes (Carrington et al. 1999, Hill et al. 1991, Thursz et al. 1997, Todd et al. 1987).

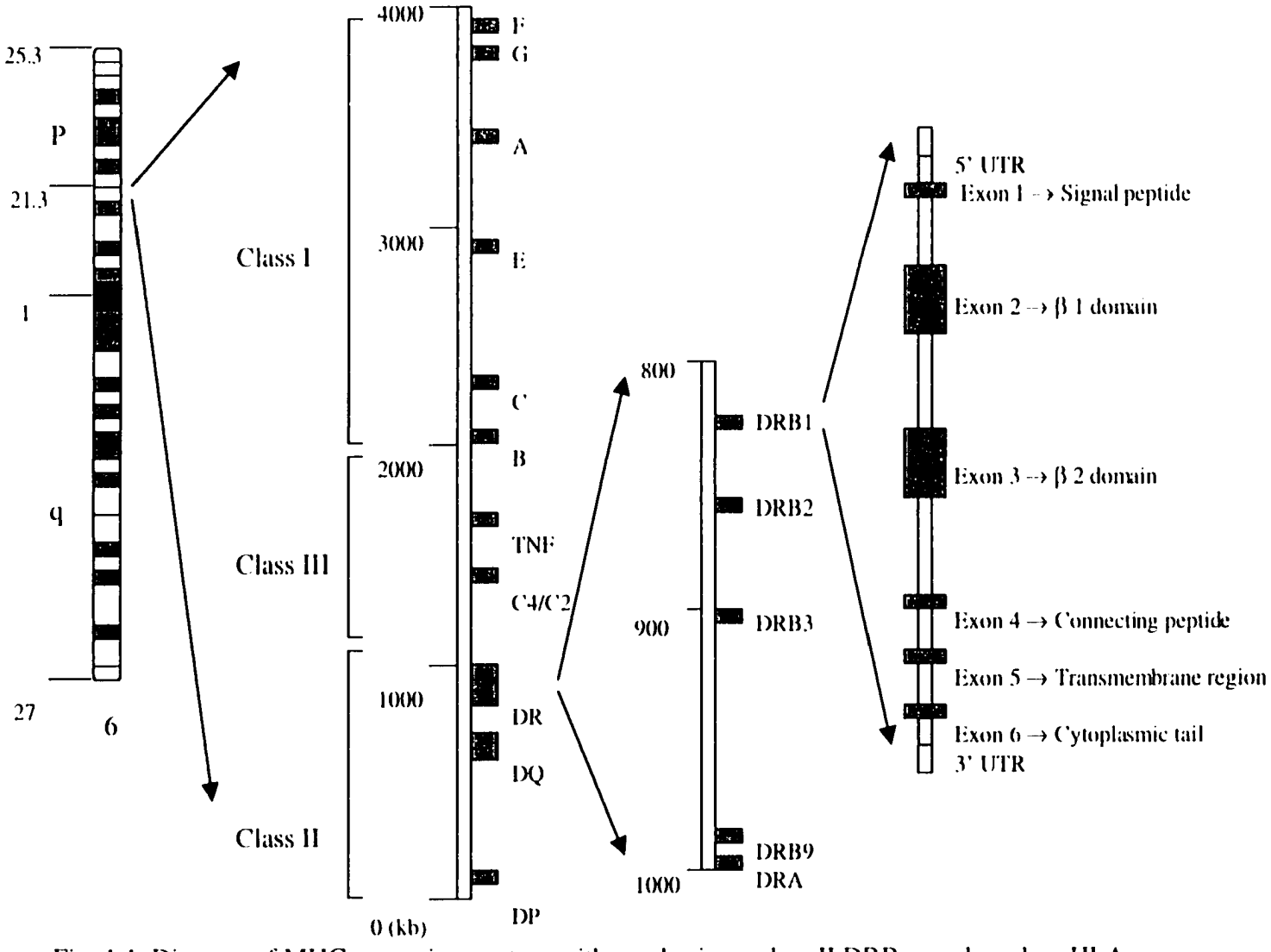

Fig. 1.1 Diagram of MHC genomic structure with emphasis on class II DRB gene based on HLA. Developed from Klein and Horejsi 1997.

The purpose of this introduction is to review some of the current knowledge about the MHC, emphasizing four aspects: structure and function, detection methods, polymorphism and evolution, and associations with disease.

Structure and function of MHC molecules:

Class I MHC molecules are glycoproteins expressed on the surface of nearly all nucleated somatic cells. The major function of these molecules is to present endogenous antigens (from virus coat proteins, for example) to the cytotoxic ($CD8^+$) T cells (T_C) (Klein and Horejsi 1997). Class I molecules are heterodimers with one large α chain (45 kDa) associated noncovalently with a much smaller β_2-microglobulin molecule (12 kDa). The α chain is encoded by the class I loci (A, B and C in HLA) on the short arm of chromosome 6 while the β_2-microglobulin is encoded on the long arm of chromosome 15. The α chain contains three external domains that are designated $\alpha 1$, $\alpha 2$, and $\alpha 3$, respectively (Fig. 1.2A). The $\alpha 1$ and $\alpha 2$ domains interact to form the peptide-binding region (PBR) that is made up of eight antiparallel β strands spanned by two long α-helical regions. This cleft is on the top of the molecule and is capable of binding a peptide of 8-10 amino acids. The $\alpha 3$ is the only domain that traverses the plasma membrane. It is highly conserved among class I MHC molecules and is recognized by the CD8 membrane molecule present on T cells. The β_2-microglobulin molecule is formed by antiparallel β strands of amino acids. It interacts extensively with the $\alpha 3$ domain and also interacts with the $\alpha 1$ and $\alpha 2$ domains.

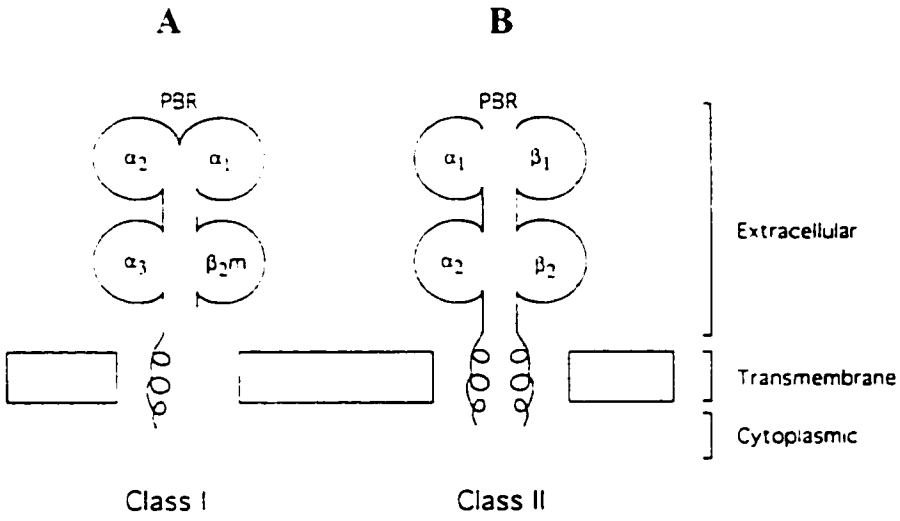

Fig. 1.2 Schematic representations of MHC class I (A) and class II (B) molecules. PBR, peptide binding region. Adopted from Hughes and Yeager, 1998.

Their interactions are essential for the class I molecules to form the correct conformation which is ultimately required for the successful antigen presentation on the surface of the cell when the class I MHC molecules bind a peptide and present it to the T_c cells (Zinkernagel 1996).

The class II MHC molecules are also membrane-bound glycoproteins but primarily expressed on professional antigen-presenting cells (APC) such as macrophages, dendritic cells and B cells. The major function of these molecules is to present the processed exogenous antigenic peptides (such as bacterial and self peptides) to helper ($CD4^+$) T cells (T_H), which respond by releasing cytokines and stimulating proliferation of B cells and cytotoxic T cells (Klein and Horejsi 1997). Like class I MHC molecules, class II MHC molecules are also heterodimers but with one α chain (33 kDa) and one β chain (28 kDa). Each chain has two domains on the surface of the molecule with $\alpha 1$ and $\alpha 2$ in the α chain, and $\beta 1$ and $\beta 2$ in β chain, respectively (Fig. 1.2B). Both chains are encoded on MHC class II loci (DP, DQ and DR in HLA) (Schafer et al. 1995). The $\alpha 1$ and $\beta 1$ domains interact to form the PBR that is capable of binding a peptide of 12-20 amino acids in length (Rudensky et al. 1991). The $\alpha 2$ and $\beta 2$ domains are both connected to the membrane of the APC through connecting peptides and have sequence homology to the immunoglobulin-fold domain structure, hence they are classified in the immunoglobulin superfamily (Williams and Barclay 1988). The α and β complex forms within the endoplasmic reticulum (ER) of the cell. Before the class II heterodimer meets any processed antigen from the endosome, a nonpolymorphic protein called the invariant chain (Ii) (31 kDa) assembles with the heterodimer, binding to the PBR. This invariant chain

mainly serves the function of blocking peptide binding in the cleft until the class II complex encounters the lower pH inside the endosome, when the invariant chain is lost from the PBR. The cleft of the heterodimer is then exposed and able to bind a processed antigen in the lumen of the endosome. Then the heterodimer with the processed antigen moves to the surface of the APC cell and is ready to elicit the activation of T_H.

Class III MHC molecules are not membrane-bound glycoproteins but secreted proteins associated with the immune process. They include serum proteins, heat shock proteins, components of complement system (e.g. C2 and C4) and tumor necrosis factors (TNF-α, TNF-β) (Fig. 1.1). Unlike class I and class II MHC molecules, class III MHC molecules are not involved in antigen presentation. Class III loci are in the middle between class II and class I loci on chromosome 6 (Trowsdale 1996).

Detection of MHC polymorphism:

As described above, the MHC gene products are heterodimeric glycoproteins that are involved in the process of presenting antigenic peptides to the cell surface. The initiation of the immune response is dependent on the recognition of antigenic peptides by the T cell receptor (TCR) (Klein 1986). In class I loci, the PBR is located on α1 and α2 domains and is encoded by the exon 2 and exon 3 region of the α chain gene. There are at least 266 different human HLA class I alleles, with the B locus most polymorphic (Parham and Ohta 1996). In class II loci, the PBR is located on α1 and β1 domains and is encoded by the exon 2 regions of the α chain and the β chain genes, respectively. There are at least 314

different HLA class II alleles, with the DRB, DQB and DPB most polymorphic (Parham and Ohta 1996). The localization of allelic polymorphism is mainly within the region that forms the antigen-binding cleft or PBR.

Many methods have been developed for detecting different MHC alleles, this is mainly due to the need for HLA matching in tissue and bone marrow transplantation, analyses of immunological function, and evolutionary investigation.

Serological typing is the typing method that has been used for decades. Because it is difficult for all laboratories to maintain and use standard reagents, discrepancies are inevitable (Deeg et al. 1982, Bunce et al. 1997). Genomic DNA restriction fragment length polymorphism (RFLP) typing method was a widely utilized technique for routine identification of HLA-DR and DQ allotypes (Howell et al. 1993). However, this method is less used now due to the fact that there are so many alleles that it is difficult to distinguish all of them.

With the application of polymerase chain reaction (PCR) techniques, several other DNA-based typing methods have been developed. Some of these methods allow rapid and efficient matching between donor and recipient and are also convenient for detection of different MHC alleles. PCR (using primers that amplify all alleles at a given locus) may be used by combining additional protocols, such as hybridization with sequence-specific oligonucleotide probe (SSOP) or exposure of the PCR amplification products to restriction enzymes and followed by display of the digestion products (Restriction Fragment Length Polymorphism, RFLP) (Sitte et al. 1996, Francino et al. 1997). These two methods take advantage of PCR as a first step to amplify enough of a specific

segment of DNA instead of merely isolating genomic DNA. Results with amplified DNA are easier to interpret than that with genomic DNA, but discrimination among all possible alleles is often ambiguous because in many cases the alleles differ in only a few bases. Another PCR-based method is to use sequence-specific primers (SSP) for the selective amplification of one or a few MHC alleles (Olerup and Zetterquist 1992). This method is based on the principle that a completely matched primer in a stringent reaction condition will be more efficiently used in the PCR reaction than a primer with one or several mismatches. Interpretation of results is based on the presence or absence of amplified products, which can be easily detected by agarose gel electrophoresis. PCR fingerprinting can be regarded as a special case of the PCR-SSP method. This method is mainly used for HLA-DR-Dw specificities. It exploits the formation of mismatched DNA hybrids from co-amplified products such as DRB genes (DRB1, DRB3, DRB4 and DRB5) in a PCR reaction. The mismatched DNA hybrids can be separated from each other on a gel by their different conformations. A similar method is single-stranded conformation polymorphism (SSCP) (Orita et al. 1989). This method uses SSP for PCR amplification and denatures the amplified PCR product prior to loading on a non-denaturing polyacrylamide gel. Single stranded DNAs have different mobilities in non-denaturing electrophoresis conditions due to minor differences in their sequences and which causes difference in secondary structures they assume during the migration. As little as a single base substitution can alter this conformation and change the mobility of the molecule. This is a simple and fast method; however, the conditions for electrophoresis are strict and may vary for different genes.

The most accurate and powerful method is DNA sequence-based typing (SBT) (Rozemuller and Tilanus 1996). This method relies entirely on an automated sequencer. Basically there are two ways of obtaining DNA sequences. One is by directly sequencing the PCR product, and the other is by cloning the PCR product followed by DNA sequencing. If a new allele is indicated, both methods need to be repeated before sending to the GenBank or for publication.

MHC polymorphism and evolution:

A remarkable feature of MHC is the common occurrence of several similar loci that form multigene and/or allelic families. Two similar models have been hypothesized to explain the formation of the MHC multigene family. One is the accordion model proposed by Klein et al. (1993a), the other is the birth-and-death model proposed by Nei et al. (1997). The essential element of these two models is the same. Both models assume that new MHC genes are created by repeated gene duplication processes during evolutionary time, and some duplicated genes are depleted or become pseudogenes due to accumulation of deleterious mutations, while others persist in the genome. The remaining duplicated genes eventually diverge from each other through accumulation of point mutations by positive selection and account for the current MHC polymorphism.

Comparative studies have shown that most species among primates, ungulates, and rodents generally have high MHC polymorphism. The high diversity is consistent with

pathogen-driven selection (the generation of MHC polymorphism is caused by the ever-changing pathogens or parasites).

Often there are several similar alleles in a species, suggesting they are derivatives of a common ancestral sequence. A given species may contain several such groups of alleles or lineages. The lineages may be shared across species, such that similarities of lineages between species are closer than different lineages within a species. The existence of such parallel lineages in related species implies that not just individual alleles but in fact the lineages (groups of similar alleles at a given locus) are inherited from a common ancestor. Jan Klein (1987) has termed this "trans-species polymorphism". The existence of trans-species polymorphism implies that much of the MHC genetic diversity in modern species is the result of retained ancestral polymorphism.

In some species, the levels of MHC variation are low. For instance, the DRB locus is the most polymorphic MHC region in most mammals, but in the African cheetah (*Acinonyx jubatus*) (O'Brien et al. 1985) and moose (*Alces alces*) (Mikko et al. 1995), the polymorphism is very low, and in European beaver (*Castor fiber*) (Ellegren et al. 1993) and musk ox (*Ovibos moschatus*) (Mikko et al. 1999), only one allele was found. In rodents, the DR locus seems to be less important than other loci since some strains do not have this locus. Moreover, the DR locus has been found functionally replaced by a DP locus in the mole rat (*Spalax ehrenbergi*) (Nizetic et al. 1987). The variation of MHC polymorphism at different loci could be due to founder effects, population bottlenecks, genetic drift, migration, and selective pressure.

In addition to the fundamental point mutation mechanism to generate allelic diversity, DNA recombination between alleles (gene conversion or interallelic conversion) has been suggested as an alternative mechanism to generate new MHC alleles (Gyllensten et al. 1991, Parham and Ohta 1996) (Fig. 1.3). Although the latter mechanism can only generate MHC diversity through reshuffling the existing allelic DNA motifs (short distinctive stretches of nucleotide sequences) accumulated by point mutations, it can rapidly and substantially increase the allelic number. It appears that the relative utilization of these two mechanisms varies with species and MHC locus (Erlich and Gyllensten 1991, Walkins et al. 1992, Mikko et al. 1995). However, Klein and O'hUigin (1995) argue that intra-locus recombination is inconsistent with both theory and observation. They proposed that similar motifs shared between different alleles could be obtained through convergent evolution (Golding 1992).

Much of the MHC polymorphism in contemporary species is almost certainly inherited from their common ancestral species according to the trans-species theory proposed by Klein (1987). As a consequence, many closely related species are found to share MHC allelic lineages (Klein and O'hUigin 1995, Graser et al. 1996, Gutierrez-Espeleta et al. 2001). An extreme case of this theory is that identical MHC alleles can be shared by two different species of primates (Evans et al. 1998, Cooper et al. 1998). The sharing of identical MHC alleles provides critical insight into the determination of how long the intact persistence of MHC alleles could be during evolution. This MHC persistence is dependent on many different factors including species, population size, selective pressure, and MHC locus.

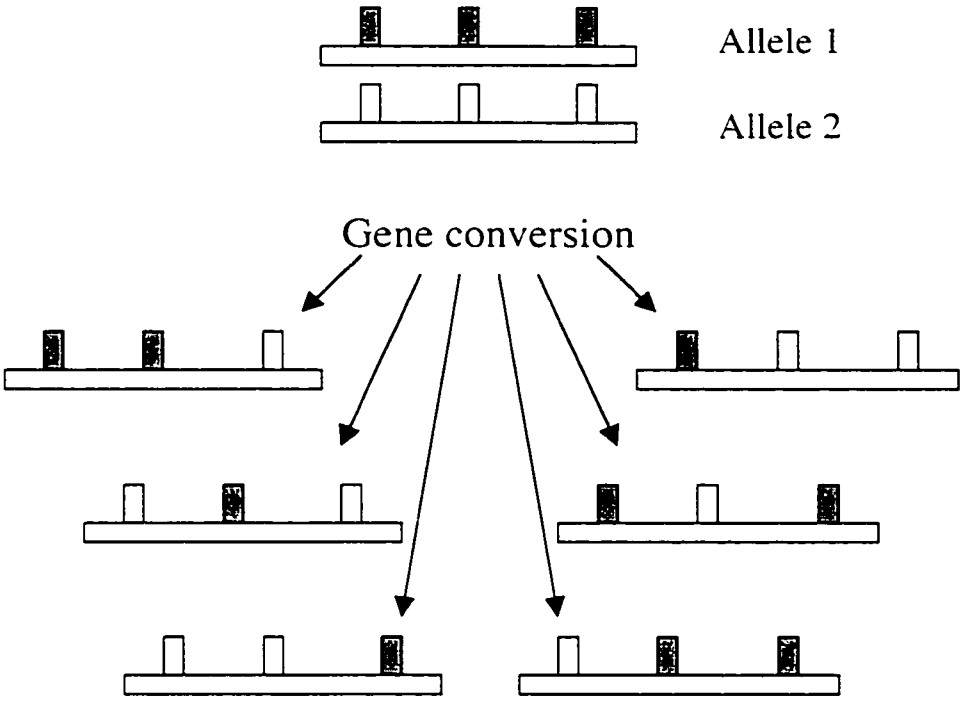

Fig. 1.3 Diagram showing how three substitutions in two alleles of the same or different MHC class II genes can generate six new alleles under the action of gene conversion. Adapted from Parham and Ohta, 1996.

Currently, there are four mechanisms that may govern the maintenance of MHC polymorphism through balancing selection have been proposed (Doherty and Zinkenagel 1975, O'Brien et al. 1985, Klein and O'hUigin 1994).

The most well accepted mechanism that maintains the MHC genetic diversity is overdominant selection (heterozygote advantage) which favors heterozygosity, allowing up to twice the number of antigenic peptides to be presented by heterozygotes as by homozygotes (Hedrick and Thomson 1983, Takahat and Nei 1990, Klein et al. 1993a) (Fig. 1.4). This diversity makes the MHC molecules able to bind a wider array of antigens, thus dealing with a broader array of infectious pathogens in the favor of hosts' survival. One of the supporting lines of evidence for the overdominant selection model is that the rate of nonsynonymous nucleotide substitutions (coding or amino acid altering) greatly exceeds the rate of synonymous nucleotide substitutions (silent or noncoding) in the codons encoding PBR (Hughes and Nei 1988 & 1989). This pattern of substitution shows that the diversity in the PBR is selectively maintained. It is the molecular evolutionary arms race (co-evolution) between MHC molecules and pathogens that lead to the MHC diversity and thus increase MHC allelic numbers as well as heterozygosity. Another strong supporting line of evidence is the observance of trans-species polymorphism (Klein 1987, Yuhki and O'Brien 1994). Balancing selection (two or more alleles are maintained at a locus), most likely the overdominant selection (heterozygotes have higher level of fitness than either homozygotes), is involved in the ancient persistence of MHC alleles. Although human and cattle have serine residue at the position 13 and 57 in DRB chain, they are encoded by different codons. This has been explained by Andersson et al. (1991) as convergent

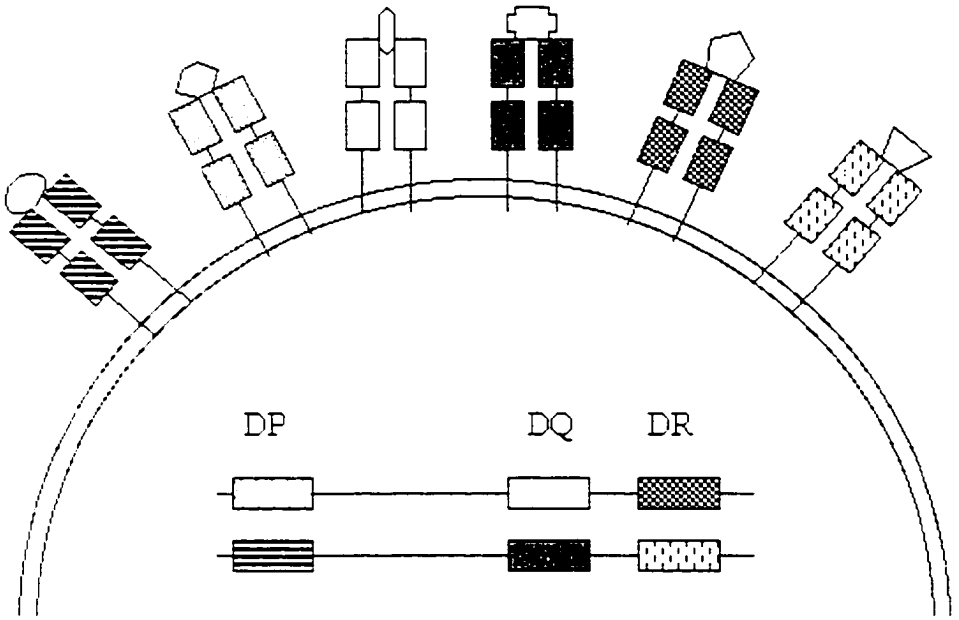

Fig. 1.4 Diagram of the heterozygote advantage of MHC class II loci. Individuals with two types of antigen-presenting molecules at each MHC class II locus (heterozygote), DP, DQ, and DR in this case, can express at least six rather than three (homozygote) different peptide-binding molecules on the cell surface. This heterozygote advantage enables T cells in the host to recognize a wider spectrum of parasites and thus allows the host to generate a stronger immune response against these invading parasites. Developed from Hill, 1999.

evolution, suggesting that the shared residues are probably due to independent evolution under the pressure of natural selection. This fact that MHC alleles from different species retain the same amino acids with different codons further indicates that MHC gene diversity is under positive selection (Takahata and Nei 1990).

Other than overdominant selection, the second most well accepted mechanism of balancing selection is frequency-dependent selection (rare allele advantage or minority advantage) (Takahata and Nei 1990, Klein et al. 1993b). This mechanism assumes that rare alleles have a selective advantage in the population and will increase in frequency. Given the important role of MHC molecules in immune surveillance, it is plausible that frequency-dependent selection is applicable to the case of MHC. When a population faces an epidemic disease, it is thought to be due to the fact that the immune system fails to provide an effective defense. One immunological defect could be that most of its alleles are unable to efficiently present the antigenic peptides of the pathogen to T cells. Thus these alleles either decrease in numbers or are lost in the population under the strong selective pressure from the pathogen. Under such conditions, if a rare allele can bind antigenic peptides strongly and efficiently, it can provide strong immunity against the pathogen and increase in frequency in the population.

The third mechanism that may maintain MHC genetic diversity is reproductive selection or MHC-based disassortative mating (Potts et al. 1991 & 1993, Penn and Potts 1998). Since MHC polymorphism is correlated with individual odors, such MHC-based disassortative mating can avoid inbreeding, increase the proportion of MHC genetic diversity, and thus increase resistance to infectious disease. Although this observation is

mainly obtained from mice, similar results have been demonstrated in some other organisms including humans (Wedekind et al. 1995, Landry et al. 2001). The most recent experiment on sticklebacks suggested that in vertebrates a female prefers the male with greater numbers of MHC alleles. The mechanism of this sexual selection is unclear but assumed through odor (Reusch et al. 2001).

The fourth suggested hypothesis is through maternal-fetal interactions (Clarke, et al. 1966). This mechanism is based on the observation that couples sharing antigens for two or three HLA-A loci have a history of spontaneous abortions. Conversely, the production of maternal antibodies to fetal MHC class I molecules is beneficial for the fetus to grow and survive. This mechanism, however, is applicable only to mammals since other organisms such as birds, reptiles, and fish do not seem to have maternal-fetal interactions. In addition, this mechanism only accounts for MHC class I polymorphism and can not explain MHC class II polymorphism. Furthermore, this mechanism has not been supported by other experiments (Wegmann 1984, Klein, 1986).

Disease associations with MHC:

The remarkable diversity of the MHC class I and class II genes is presumably maintained to increase the ability of a species to fight potential pathogen intruders by presenting a wide array of antigens and thus generate diverse immune responses (Doherty and Zinkernagel 1975). Within a species, however, some individuals may have a set of MHC alleles that are more resistant to a given pathogen, while others may have a quite

different set of alleles that are more susceptible to a given pathogen during disease epidemics. Once the number of alleles decreases due to a genetic bottleneck or to inbreeding, the ability to resist diverse pathogens may also be decreased. There are several such examples for endangered species such as the cheetah, whale, and southern elephant seal (O'Brien et al. 1985, Trowsdale et al. 1989, Slade 1992) which show low diversity and are thought to be disease susceptible. In humans, a good example may be the death of Amerinds in the exposure of diseases introduced by explorers from the Old World (Black 1992).

Large-scale studies of HLA class I antigens and their association with human viral diseases have shown that there is a statistically strong link between HLA class I alleles and the resistance or susceptibility to a given virus. Jeffery and colleagues (1999) detected a common HLA class I allele HLA-A*02 protective association with human T-lymphotropic virus-1 (HTLV-1). This allele was found to be associated with a greater than two-fold reduction in the risk of disease induced by HTLV-1. The frequency of this allele was found constantly lower in patients with disease than in infected people without disease, and the viral load was much less in people with this allele. The authors believed that the protective role of this allele could be due to the high levels of virus-specific T cells detected in the healthy carriers of HTLV-1 (Jeffery et al. 1999). Another example is the heterozygote advantage in the protection of acquired immunodeficiency syndrome (AIDS). Carrington et al. (1999) reported that two other HLA class I alleles, B*35 and Cw*04, are found to be consistently associated with rapid AIDS progression among Caucasians after infection with the human immunodeficiency virus-1 (HIV-1).

Maximum heterozygosity at HLA class I loci (A, B, and C) protected AIDS progression among patients infected with HIV-1, while people who were homozygous for one or more loci made a rapid progression to AIDS (Carrington et al. 1999). Some other diseases such as malaria and hepatitis B have also been documented in association with HLA class I alleles (Gilbert et al. 1998 and Thurz et al. 1997).

For MHC class II alleles, a significant association of enzootic bovine leukosis (EBL), a disease induced by the infection of bovine leukaemia virus (BLV), has been reported with some of bovine lymphocye antigen (BoLA) class II haplotypes in cattle (Zanotti et al., 1996). One haplotype, DQA*3A-DQB*3ADRB2*2A-DRB3.2*11, was found to be associated with resistance to this disease, while the other haplotype, DQA*12-DQB*12-DRB2*3A-DRB3.2*8, was found to be associated with susceptibility to this disease. A similar result was also found in unmanaged Soay sheep populations showing that MHC variation is significantly associated with differences in juvenile survival and resistance to intestinal nematodes (Paterson et al. 1998).

As proposed by Klein and O'hUigin (1994), the MHC polymorphism has been strongly influenced by parasites coevolving with their hosts during evolutionary time. In contrast, the recent parasites have little effect on the MHC polymorphism due to the lack of enough time to establish selective relationship between the hosts and the parasites.

The investigation of the MHC allele associations with resistance or susceptibility to different diseases is important for the precise understanding of host-parasite co-evolution and its effect on the maintenance of MHC polymorphism.

Hypothesis and goals:

The dramatic climate changes during the Quaternary (2.4 million years to the present) had a great influence on the evolution and distribution of contemporary species, especially in the Arctic region (Hewitt, 2000). From a genetic point of view, the consequences of these ice ages on the extant taxa have been difficult to determine and such studies have been limited due to lack of appropriate materials and genetic markers (Leonard et al. 2000).

According to Klein (1994), there are two worlds that MHC has to deal with. One is the self-world, the other is the non-self world. To the self-world, MHC needs to keep its stability which allows its gene products to be recognized by T cells. To the non-self world, MHC favors its instability which generates additional MHC alleles that can help the host fight the ever-changing parasites. In order to keep its instability, MHC needs to be under some kind of natural selection, presumably through the balancing selection that might be driven by the ever changing parasites (Klein 1987, 1994). However, in the Arctic region, such selection pressure by parasites may be low and specific due to the extreme cold weather and simple ecosystem that greatly reduces the parasite spectrum. The narrowed parasite spectrum thus allows the relatively stable relationships established between hosts and parasites. As suggested by Geist (1985), northern species might have lost resistance to particular parasites which are present at lower latitudes but absent at high latitudes and thus the high latitude species are vulnerable to infectious diseases when they meet other southern species, particularly members of their own lineage. In fact, several studies have

already shown that some northern species, such as moose, musk ox, and polar bear, have very low levels of genetic variation at some MHC and mitochondrial genetic markers (Mikko et al. 1995 & 1999, Ellegren et al. 1996, Groves et al. 1997, Shields and Kocher 1991). On one hand, with low selection pressure the process of antagonistic co-evolution between hosts and parasites could be slow, allowing northern species to have a good chance to lose many MHC alleles inherited from their recent ancestor(s) mainly through random genetic drift and population bottlenecks. On the other hand, with specific selection pressure the relationships between hosts and parasites are stable and some MHC alleles may be persistent with the hosts driven by specific parasites, allowing some MHC alleles to be shared by different species.

This thesis investigates the hypothesis that reduced natural selective pressure in the Arctic region, due to the narrow spectrum of parasites caused by the harsh weather, may allow northern species to have generally low levels of MHC variation after speciation (Geist 1985, Hewitt 2000) and have a very similar MHC allelic patterns to other closely related species.

In order to test the hypothesis, two groups of northern species were chosen. One group includes the polar bear and the moose that have a solitary life style, the other group includes the musk ox and caribou (reindeer) which live in small and large herds, respectively. As there are well documented records indicating that musk ox experienced genetic bottlenecks, a man-made genetic bottleneck in the Doberman pinscher dog lineage is included in this investigation to explore the effect of artificial selection on the maintenance of MHC polymorphism.

Several specific goals of this study were:

1. To investigate which MHC class II locus is maintained preferably in musk ox and moose by assessing levels of MHC variation at the DRB and DQB loci, since both species showed either monomorphism or very low level of variation at the DRB locus;
2. To characterize the MHC polymorphism at the DRB locus in one species, *Rangifer tarandus*, between domestic (reindeer) and wild (caribou) populations;
3. To assess the level of MHC diversity locus in polar bears at the most polymorphic DRB. This will be the first of such studies in bears which can provide genetic information of MHC variation impacted by the arctic harsh weather on arctic carnivores, and allow the comparison of MHC polymorphism between herbivores and carnivores in the Arctic;
4. To compare the genetic bottleneck effect on the maintenance of MHC variation between a natural bottlenecked population (in musk ox) and the man-made one (in a Doberman pinscher dog lineage).

The arrangement of the chapters is as follows:

- Chapter II confirms the monomorphism of the DRB locus in musk ox (*Ovibos muschatus*) and focuses on the comparison of DQB polymorphism between the musk ox (*O. muschatus*) and the moose (*Alces alces*);
- Chapter III characterizes MHC DRB allelic variation in domestic and wild populations in *Rangifer tarandus*, and analyzes its phylogenetic relation among cervid DRB alleles;

- Chapter IV describes the MHC variation at the DRB locus in the polar bears (*Ursus maritimus*);
- Chapter V characterizes the MHC variation at both DRB and DQB loci in a Doberman pinscher dog lineage;
- The last chapter is Chapter VI which gives concluding remarks and future perspectives.

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Chapter II

Comparison of MHC genetic diversity at the class II loci between musk ox (*Ovibos moschatus*) and moose (*Alces alces*)[1]

Abstract

Objective: Since both musk oxen and moose exhibit low levels of major histocompatibility complex (MHC) genetic variation at DRB locus, one purpose of this study is to confirm the musk ox DRB monomorphism with an expanded sample size and examine its DNA expression. The other purpose of this study is to compare MHC genetic diversity at other class II loci, particularly at the polymorphic DQB locus, between the two arctic ungulates of musk ox and moose.

Methods: Exon 2 regions of MHC class II loci genes were amplified by PCR. Allelic polymorphism was analyzed using single-stranded conformation polymorphism (SSCP), DNA cloning, and DNA sequencing. Gene expression was detected by cDNA sequencing. Phylogenetic analysis was done with PAUP and MEGA.

Results: Monomorphism was found in musk oxen at DRA, DRB, and DQA loci. The monomorphic DRB seems to be functional since the full-length cDNA was transcribed. The DQA has a synonymous substitution (G/A) at the 192 nucleotide position which correlates well with the proposed definition of the two main musk ox subspecies. In addition, 9 DQB alleles plus 2 DQB pseudogenes were found in musk ox. For the

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moose, only one allele at DRA was detected, while two moose DQA alleles were found. One moose DQA allele showed identity with one of the musk ox DQA alleles, and a second moose DQA allele was quite different with 20 nucleotides leading to 10 amino acid changes. This allele was only found in one sample obtained from Russia. Four moose DQB alleles and 2 DQB pseudogenes were detected. The 4 moose DQB alleles were identical to musk ox DQB alleles. Moreover, the predominant DQB pseudogene was shared by these two species as well. The other DQB pseudogene in each species seems to be the result of interallelic recombination between one of the functional alleles and the predominant DQB pseudogene, respectively.

Conclusion: The sharing of identical DQB and DQA alleles between two distantly related species such as musk ox and moose suggested that similar selection pressures might be acting in parallel on the DQ loci of both species in the Arctic. Some MHC genes could be used as genetic markers to address population questions.

Key words: MHC class II loci, musk ox, moose, trans-species polymorphism, substitution rate, genetic marker

Data deposition: The sequences reported in this study have been deposited in the GenBank database (accession nos. AY077784-AY077796).

Introduction:

The major histocompatibility complex (MHC) exhibits an extraordinary high level of genetic variation in most vertebrate species (Klein 1986). Two classes of MHC molecules are involved in the presentation of antigens. Class I molecules present endogenous antigens to $CD4^+$ cytotoxic T lymphocyes (CTL), while class II molecules present extrogenous antigens to $CD8^+$ helper T lymphocytes. The immunological role of the antigen presentation by MHC molecules is to mount a defensive system in the host against the infections by pathogen invaders (Doberty and Zinkernagel 1975). It is predominantly the antigen-binding site (ABS) residues on the MHC molecules that show MHC variability (Bennet et al. 1987, Brown et al. 1988 and 1993). This MHC genetic variability is presumably maintained by some form of balancing selection (Hedrick and Thomson 1983), most likely overdominant selection which prefers heterozygosity (Hughes and Nei 1988 and 1989). MHC heterozygosity permits MHC molecules to present a broader range of antigenic peptides, leading to stronger immune responses against invading parasites.

The parasite-driven hypothesis is based on the notion that the ever-changing parasite is the driving force for the MHC polymorphism (Klein and O'hUigin 1994). Other selective forces, such as negative assortative mating and maternal-fetal interaction, have also been proposed (Potts et al. 1991 & 1993, Reusch et al. 2001, Clarke, et al. 1966). According to Klein (1987), much of the MHC polymorphism in contemporary species could be inherited from their recent common ancestral species, having been

passed from one species to another during the evolution. As a consequence, many MHC alleles from different species are more similar than those even from the same species, forming an allelic lineage. This phenomenon is termed trans-species polymorphism. It has been suggested that species with low levels of MHC diversity may be vulnerable to various kinds of infectious diseases (O'Brien and Evermann 1988).

The post-Pleistocene ice ages (1.8 million to 11,000 years ago) have had great genetic impacts on contemporary species in the Arctic (Kurten and Anderson 1980, Hewitt 2000). This could be attributed to climatic changes, population bottlenecks, migrations, adaptations, and natural selection. Both musk ox (*O. moschatus*) and moose (*A. alces*) entered North America through the Bering Land Bridge from Siberia into unglaciated refugia in Alaska. The earliest appearance of musk oxen in North America is estimated at around 90,000 years ago (Klein 2000), while the appearance of moose is less than 70,000 years ago during the Wisconsin glaciation (Franzmann 2000). These two species are both circumpolar herbivorous ungulates that have adapted well to the harsh weather in the Arctic region. Similar habitat and life style of these two species provides a good opportunity to compare the evolution of MHC polymorphism.

Previous studies showed that both species have low levels of genetic variation. Muskoxen showed extremely low levels of genetic diversity in allozyme profiles, mitochondrial DNA, and microsatellite DNA analyses (Fleischman 1986, Groves 1997, and Holm et al. 1999). At the MHC class II DRB locus, only one allele was detected (Mikko et al. 1999). Previous MHC studies in moose also showed low levels of genetic diversity at both class I and class II loci (Elllegren et al. 1996). For the class II loci, the

DRB gene was investigated in detail by using SSCP and nucleotide sequencing, and very limited polymorphism of DRB was found (Mikko et al. 1995).

The objectives of this study were (i) to characterize musk ox MHC genetic variation at DRA and DRB loci by expanding the sample size; (ii) To compare the MHC polymorphism at the DRA, DQA and DQB loci between musk ox and moose.

Materials and methods:

Animals:

Blood samples were obtained from all major musk ox populations worldwide. Localities were geographically well distributed, including Greenland, Bank Island, Victory Island, mainland Canada (Coppermine) and Alaska. The total sample size is 65. Twenty-one moose blood samples were also included in this study. Among these samples, 20 were collected from different localities in Alaska and one from Siberia (provided by the Museum of University of Alaska). Genomic DNA was extracted using QIAamp blood kit (Qiagen, Chatsworth, California) according to the manufacturer's instructions.

PCR, SSCP, and DNA sequence analysis:

Primers (Operon) and conditions for polymerase chain reaction (PCR) are described in Table 2.1. The PCR amplification was performed with 5 µl of genomic DNA (200–400 ng) as template in a 50 µl reaction containing 5 µl of 10 × buffer, 5 µl of

magnesium chloride (12.5 mM), 2 µl of 4 × dNTPs (2.5 mM), 1 µl of each primer (10 pmol), and 0.5 µl of *Taq* Polymerase (2 units). The PCR program consisted of an initial 3 min at 95°C, then total 35 cycles of 95°C for 30 s followed by corresponding annealing temperature (Table 2.1) for 30 s and 72°C for 1 min, and a final 10 min extension at 72°C. All PCR reactions were performed in 96-well plates using a GeneAmp PCR System 9700 (PE/Applied Biosystems). PCR products were monitored by running a 5 µl sample on a 2% agarose gel stained with ethidium bromide, and were purified either by QIAquick PCR purification columns for direct sequencing or by a QIAquick gel extraction kit (Qiagen) for cloning.

SSCP analysis was performed essentially according to the method described by Clay et al. (1995). Briefly, 10-15 µl of PCR product was mixed with 3 µl loading buffer containing denaturing solution (95% formamide, 20 mM EDTA and 0.01% bromophenol blue, pH 8.0), and was heated at 97°C for 5 min, rapidly cooled on crushed ice for 5-10 min and then loaded on a 12.5% non-denaturing polyacrylamide gel (acrylamide:bis-acrylamide=50:1 in 0.5xTBE). Electrophoresis was performed at 150 V for 50 min at a constant temperature of 20°C. Gels were stained in 0.5 µg ml^{-1} ethidium bromide for 20 min and then visualized on an UV transilluminator. Typical SSCP allelic fragments were isolated from the gel by cutting these fragments from the gel stained by ethidium bromide. Gel fragments were put into 50 µl of sterile water and crushed in a 1.5 ml tube by using a sterile pipette tip. The tube was then put in a 4°C refrigerator overnight. The supernatant was desalted by QIAquick PCR purification column and 5 µl was used as template in a second round of PCR.

The PCR product was used for DNA sequencing after purification. DNA sequencing on both strands was carried out on an ABI 373A DNA Sequencer or ABI 3100 Genetic Analyzer using fluorescent dye terminators (Applied Biosystem Inc.). All samples were used either for SSCP analysis or for the first round of direct sequencing of PCR products. For homozygous samples, PCR products were then used for the second round of direct DNA sequencing. For heterozygous samples, however, PCR products were cloned using Perfectly Blunt Cloning Kits (Novagen, Madison, Wisconsin). Plasmid DNA was purified from white clones using Wizard Plus Minipreps (Promega, Madison, Wisconsin). An average of 6-10 clones were selected and sequenced for each sample. Alleles that repeated at least twice in different animals were used for further analysis.

cDNA analysis:

mRNA was prepared from blood using the RNeasy Mini Kit (Qiagen). First strand cDNA was synthesized using the Reverse Transcription System (Promega) with random primers according to the manufacturer's instructions. Ten out of 20 µl of the first strand cDNA product was used as the template for the first round of PCR amplification. Primers and conditions for the cDNA PCR are described in Table 2.1. PCR was performed in a thermocycler (GeneAmp PCR system 9700, Applied Biosystems) with an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, corresponding annealing temperatures for 30-60 s, and 72°C for 30-60 s, accordingly. Five µl of the first round of PCR product (DQBcF and DQBcR2) was then used as the template for the

second round of PCR amplification (DQBcF and DQBcR1) for the DQB cDNA (Table 2.1). The amplified second round of PCR product was purified and sequenced on both strands as described as above.

Microsatellite markers:

MHC variation was also assayed using three pairs of polymorphic microsatellite primers that are located within sheep MHC class I and class II regions (Paterson et al. 1998). One of the primers from each pair was synthesized (Operon) with a fluorescent dye group, either FAM, TET, or HEX (ABI), at the 5' end. The size of microsatellite fragments was detected on an ABI 373A DNA sequencer using the GeneScan software (ABI).

Phylogenetic analysis:

Phylogenetic trees were constructed using the computer program PAUP 4.0 (Swofford, 2000) carrying out a heuristic search performing tree-bisection-reconnection (TBR) branch swapping by applying genetic distances estimated with Kimura's two-parameter method (Kimura, 1980). Pairwise comparison of nucleotide substitutions between alleles were conducted by the computer program MEGA (Kumar et al. 1993) according to the method of Nei and Gojobori (1986). The ratio between nonsynonymous (dN) and synonymous substitutions (dS) were estimated applying Jukes and Cantor's (1969) correction for mutltiple hits.

Results:

DR loci: Exon 2 of DRA and DRB loci was successfully amplified by PCR using caprine-specific primers (Table 2.1) from musk ox genomic DNA.

For DRA, polymorphism was investigated by sequencing exon 2. Only one allele was detected in all the samples tested (Fig. 2.1). SSCP analysis revealed only one allelic type represented by three distinct SSCP bands for the DRB locus (Fig. 2.2). Each of these three SSCP bands was then confirmed by DNA sequencing, either by directly sequencing the PCR products or by sequencing the DNA fragments recovered from the SSCP gel (Fig. 2.2). Both DRA and DRB sequences were also confirmed by cloning the PCR products followed by DNA sequencing.

To test whether the DRB locus is transcribed or not, both exon 2 and the full-length cDNA were successfully amplified by PCR (Table 2.1) for subsequent sequence analysis. The sequence of exon 2 cDNA turned out to be identical to that defined by PCR analysis of genomic DNA (Fig. 2.3), which indicated the expression of the monomorphic DRB exon 2. Furthermore, the expression of DRB was confirmed by sequencing the full-length cDNA (Fig. 2.4). The deduced amino acid sequence from the musk ox DRB was compared with several typical mammalian species including three ruminant species, cattle, sheep and goat (Fig. 2.5).

To clarify the genetic relationships of the expressed DRB genes among the cattle, sheep, goat, and musk oxen, a maximum parsimony phylogenetic tree was generated using their full-length cDNA nucleotide sequences. The musk ox DRB is located in the

same lineage as those from cattle, sheep, and goat (Fig. 2.6), which is consistent with the recent phylogeny based on nuclear DNA showing that musk ox is very close to sheep and goat (Matthee and Davis, 2001)

This study examined DRA in all samples tested and found only one moose DRA allele which is different from the DRA allele of musk ox (Fig. 2.7).

DQ loci: Musk ox DQA exon 2 was sequenced by both PCR and cloning methods. Only one allele was found in all samples tested, but there was a synonymous substitution at the nucleotide position 192 of the DQA exon 2 (Fig. 2.8). Interestingly, this synonymous substitution of DQA exon 2 could be used as a genetic marker to distinguish samples originally from mainland Canada populations (nucleotide A at 192 position in the DQA exon 2) and samples originally from Greenland populations (nucleotide G at the 192 position in the DQA exon 2) (Table 2.2). Moreover, samples from Banks Island, Canada, showed both alleles including several heterozygous samples.

In contrast to the monomorphism of DRB, musk ox DQB showed relatively higher polymorphism with a total of 9 alleles and 2 pseudogenes detected (Fig. 2.9 & Fig. 2.10). The musk ox DQB alleles seem to be functional since one of the alleles, Ovmo-DQB*01, was determined from the cDNA using primers from highly conserved parts of DQB exon 2 and 3 (Fig. 2.11). In addition, the estimated rate of nonsynonymous (dN) was greater than that of synonymous (dS) among these musk ox DQB alleles (Table 2.3), suggesting that positive selection has been applied on these alleles. As for the moose, 2 DQA alleles were found from all samples examined (Fig. 2.12). One moose DQA allele (DQA*01)

showed identity with one musk ox DQA allele, the other (DQA*02) was obtained only from the single Russian sample. For the moose DQB locus, 4 DQB alleles plus 2 pseudogenes were detected (Fig. 2.13 & Fig. 2.14). One of the moose DQB alleles, Alal-DQB*03, was found to be transcribed by determining its sequence from the cDNA (Fig. 2.15). It is quite likely that there were two functional DQB loci in the moose because one of the individual samples was found to contain four different DQB alleles. Interestingly, one of the pseudogenes (DQBΨ*02) seems to be the result of interallelic recombination between one functional allele and the major pseudogene (DQBΨ*01) (Fig. 2.10 & Fig. 2.14). Furthermore, four DQB alleles and the major pseudogene (DQBΨ*01) between musk ox and moose were found to be identical at the nucleotide level (Fig. 2.16), indicating a trans-species polymorphism between these two species.

Microsatellite markers: Three sets of microsatellite primers derived from sheep were tried in this study to detect the genetic diversity within musk ox MHC region (Paterson et al. 1998). Two pairs of them designed to amplify microsatellite markers located within MHC class II DRB region failed. The third pair of primers which is located within the MHC class I region, however, was used successfully. For this microsatellite marker, 61 out of 65 (94%) samples showed homozygosity, only 4 samples (6%) showed heterozygosity. This result demonstrated that there was low genetic diversity within the musk ox MHC class I region.

Discussion:

In this study, we compared the genetic variabilities of the MHC class II at DR and DQ loci in musk ox (*O. moschatus*) and moose (*A. alces*). We found one identical MHC DQA allele and four identical MHC DQB alleles shared by these two species.

Our results confirmed the previous reported monomorphism of the DRB in the musk ox with our larger sample size (Mikko et al. 1999), and also showed MHC monomorphism at DRA locus in musk ox. In addition, one musk ox DQA allele with a synonymous mutation was also detected. A low degree of variation was also found in the class I region using a microsatellite marker. In contrast, our data also showed that musk ox has a relatively high polymorphism at the DQB locus with 9 alleles and 2 pseudogenes (Fig. 2.9 & Fig. 2.10), implying that the DQB locus may be under stronger diversifying selection pressure. In general, except for the DQB, these results are consistent with previous reports of low genetic diversity which include the low allozyme variation (Fleischman 1986), the low mitochondrial DNA variation (Groves 1997), and the low microsatellite DNA variation (Holm et al. 1999).

The previous study of moose DRB demonstrated 10 alleles with a very limited genetic variability (Mikko et al. 1995). This present study extended the investigation at DRA, DQA, and DQB. Data from this study showed only one DRA allele (Fig. 2.7), two DQA alleles with one obtained from the Russian sample (Fig. 2.12). There were 4 moose DQB alleles and 2 DQB pseudogenes (Fig. 2.13 & Fig. 2.14).

One of the moose DQA alleles was found to be identical with one of the musk ox DQA alleles (Fig. 2.12). In addition, 4 moose DQB alleles were found to be identical with 4 musk ox DQB alleles as well. Surprisingly, the most frequent DQB pseudogene was shared by these two species (Fig. 2.16). We found identical MHC class II DQA and DQB alleles shared by two distantly related species and their lineages split approximately 23 million years ago (Vrba, 1985, Novacek 1992, Kumar and Hedges 1998).

Genetic bottlenecks have been hypothesized to explain for the low level of genetic variation in musk ox (Grove 1997, Holm et al. 1999). However, for moose, an genetic bottleneck which occurred sometime in the last 10,000 to 50,000 years (before the divergence of subspecies) has been proposed to explain the reduced MHC diversity (Ellegren et al. 1996, Mikko et al. 1995).

A synonymous substitution at the musk ox DQA locus can be used to separate two main musk ox populations which represent two major subspecies (Groves 1997), the barren-ground muskox (*O. moschatus moschatus*) and the white-faced muskox (*O. maschatus wardi*) (Fig. 2.8). These two musk ox populations may have mixed on the Banks Island and are likely on other islands such as Victory Island (Table 2.2). The correspondence between the synonymous substitution at DQA gene and the two musk ox subspecies makes the DQA a perfect genetic marker to distinguish the current musk ox populations. Moreover, this correspondence allowed us to determine when this synonymous mutation might have occurred. The formation of the two subspecies has been suggested during the Wisconsin glaciation period which is dated back to 70,000 to 10,000 years BP (Groves 1997). Therefore, the fixation of this synonymous mutation is

at least that long. This allowed us to calculate the population size before this mutation fixed in the musk ox population. According to the method of Kimura and Ohta (1969), the mean conditional fixation time t can be calculated with the following equation: $t = 4N$ generations, where N represents population size. If we put the time scale of 70,000 to 10,000 years into the equation and assume the average generation time for musk oxen is 5 years, the musk ox population size N during the last glaciation could be 500 to 3,500 individuals. As for the moose, the DQA*02 allele which is obtained only from the Russian sample implies that this allele may be used to distinguish moose populations from Alaska and Russia (Fig. 2.12).

A prediction of the trans-species polymorphism (Klein 1987) is that similar or identical MHC alleles may be shared by closely related species. Numerous previous studies of MHC polymorphism have shown no shared alleles among species, except two recent studies on primates. These two recent studies have found that a MHC class I allele is shared by two primate species which split 0.7 and 2.3 million years ago, respectively (Evan et al. 1998, Cooper et al. 1998). The finding that identical MHC class II DQA and DQB alleles were shared by the musk oxen and moose in this study provides the first example of strict MHC trans-species polymorphism between individual species in families which diverged approximately 23 million years ago (Fig. 2.12 & 16, Vrba, 1985, Novacek 1992, Kumar and Hedges 1998). Specific and persistent positive selection produced by common shared pathogens in the Arctic might have involved in the process of keeping identical MHC alleles in different species.

Musk ox and moose both have had limited histories in North America with a time scale shorter than 9,000 years (Klein 2000, Franzmann 2000). The mutation rate of MHC class II genes is actually slow, the average rate for synonymous substitution is 1.18×10^{-9} substitutions per site per year, and it may take up to 1.6 million years to accumulate one substitution into a gene (Klein et al. 1993). Thus, it seems unlikely for different MHC alleles to converge into an identical one, leading to the sharing of alleles by the musk ox and moose. In fact, the distinct moose DQA allele obtained only from the Russian sample implies that the Alaskan population may be different from the Russian population. However, the same Russian sample contains the moose DQB*01 and DQB*03, both alleles are identical with the musk ox DQB*01 and DQB*08, respectively (Fig. 2.16). This indicates that the shared alleles between the musk ox and moose existed at least in the moose before their first appearance in North America. Similarly, it is difficult to explain that the shared DQA and DQB alleles in the musk ox are through convergent evolution, since there seems no enough time for the musk ox to accumulate so many mutations. One possible scenario could be that both the musk ox and moose inherited these shared identical alleles from their recent common ancestor and kept them ever since. These MHC alleles may have established a stable co-evolutionary relationship with some parasites specific to the musk ox and moose. This kind of positive selection may help these shared MHC alleles pass through many speciation events to be presented in the current musk ox and moose populations. The musk ox and moose are both arctic species and have a circumpolar distribution. The similar habitat and isolated

lifestyle of these two species may provide another line of evidence to explain the shared MHC alleles.

Of course, the persistence of these shared DQB alleles between the musk ox and moose does not exclude the possibility of accumulating mutations. In fact, some of the musk ox DQB alleles differ only by one or two amino acid substitutions such as Ovmo-DQB*01, Ovmo-DQB*03, Ovmo-DQB*04 and Ovmo-DQB*06, which demonstrated the possibility that the Ovmo-DQB*03, Ovmo-DQB*4, and Ovmo-DQB*06 may be developed from the most frequent Ovmo-DQB*01 allele by the accumulation of point mutations, respectively. In contrast to the monomorphic musk ox DRB allele which may be under strong purifying selection, the musk ox DRB locus may be under strong positive selection, demonstrating the usage preference of MHC loci in the musk ox (Nizertic et al. 1987).

Recombination events may also be involved in the generation of DQB alleles as well, since the DQB*Ψ02 in the musk ox and moose, respectively, was found to be likely the results of interallelic recombination between one functional allele and the predominant pseudogene, respectively (Fig. 2.10 & Fig. 2.14).

```
AATCATGTGATCATCCAAGCTGAGTTCTATCTGAACCCTGAGCAATCAGCC   51
 N  H  V  I  I  Q  A  E  F  Y  L  N  P  E  Q  S  A

GAGTTTATGTTTGACTTTGATGGTGATGAGATTTTCCACGTGGATATGCAG  102
 E  F  M  F  D  F  D  G  D  E  I  F  H  V  D  M  Q

AAGAAGGAGACAGTGTGGCGGCTTCCAGAATTTGGACATTTTGCCAGCTTT  153
 K  K  E  T  V  W  R  L  P  E  F  G  H  F  A  S  F

GAGGCTCAGGGTGCCCTGGCCAATATGGCTGTGATGAAAGCCAACCTGGAC  204
 E  A  Q  G  A  L  A  N  M  A  V  M  K  A  N  L  D

ATCATGATAAAGCGCTCCAACAACACCCCAAACACCAATG              244
 I  M  I  K  R  S  N  N  T  P  N  T  N
```

Fig. 2.1 Nucleotide sequence and its deduced amino acid of the monomorphic musk ox DRA exon 2. Underlined nucleotides are two primers, respectively.

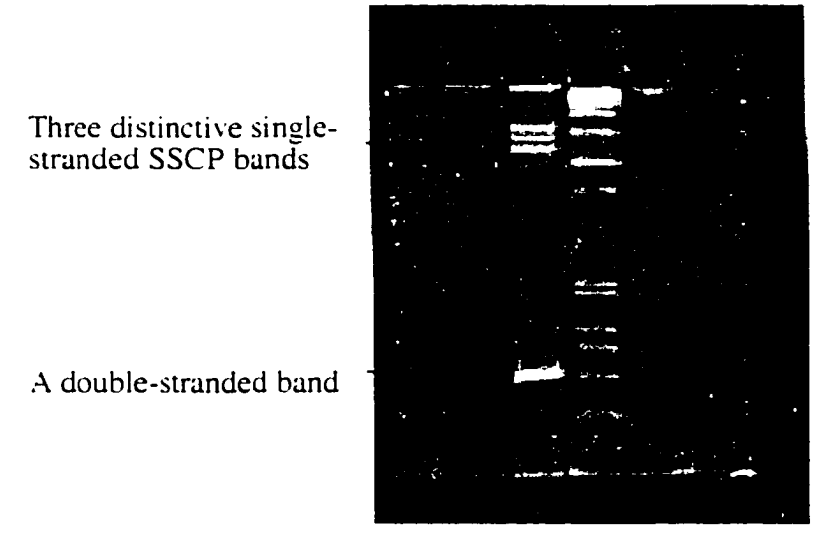


Fig. 2.2 SSCP bands of a musk ox sample on a 12.5% polyacylamide gel. All samples tested showed three distinctive SSCP bands. The number 15 represents a sample and the M is a DNA size marker.

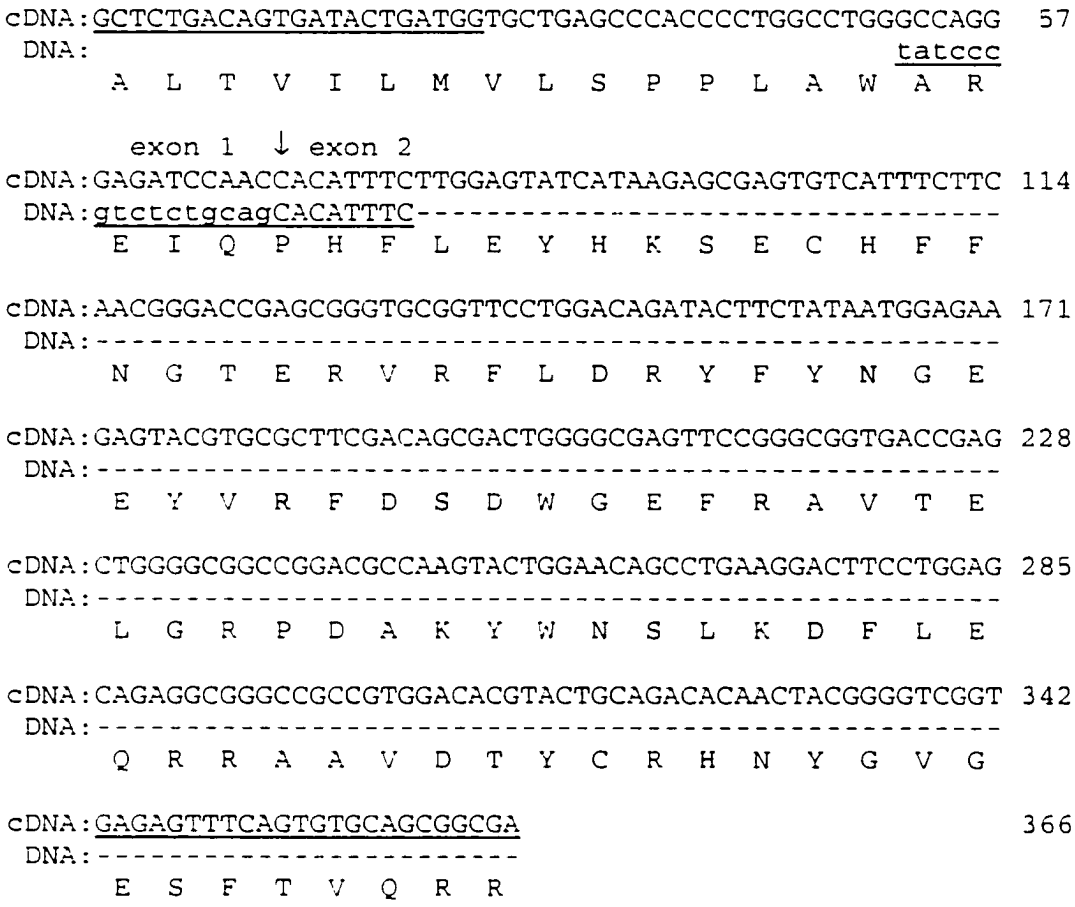


Fig. 2.3 Comparison of genomic and cDNA sequences obtained from the musk ox DRB exon 2 region. Nucleotides showed in lower case represent intron sequence. The border between exon 1 and exon 2 is indicated by a vertical arrow. The primers are underlined.

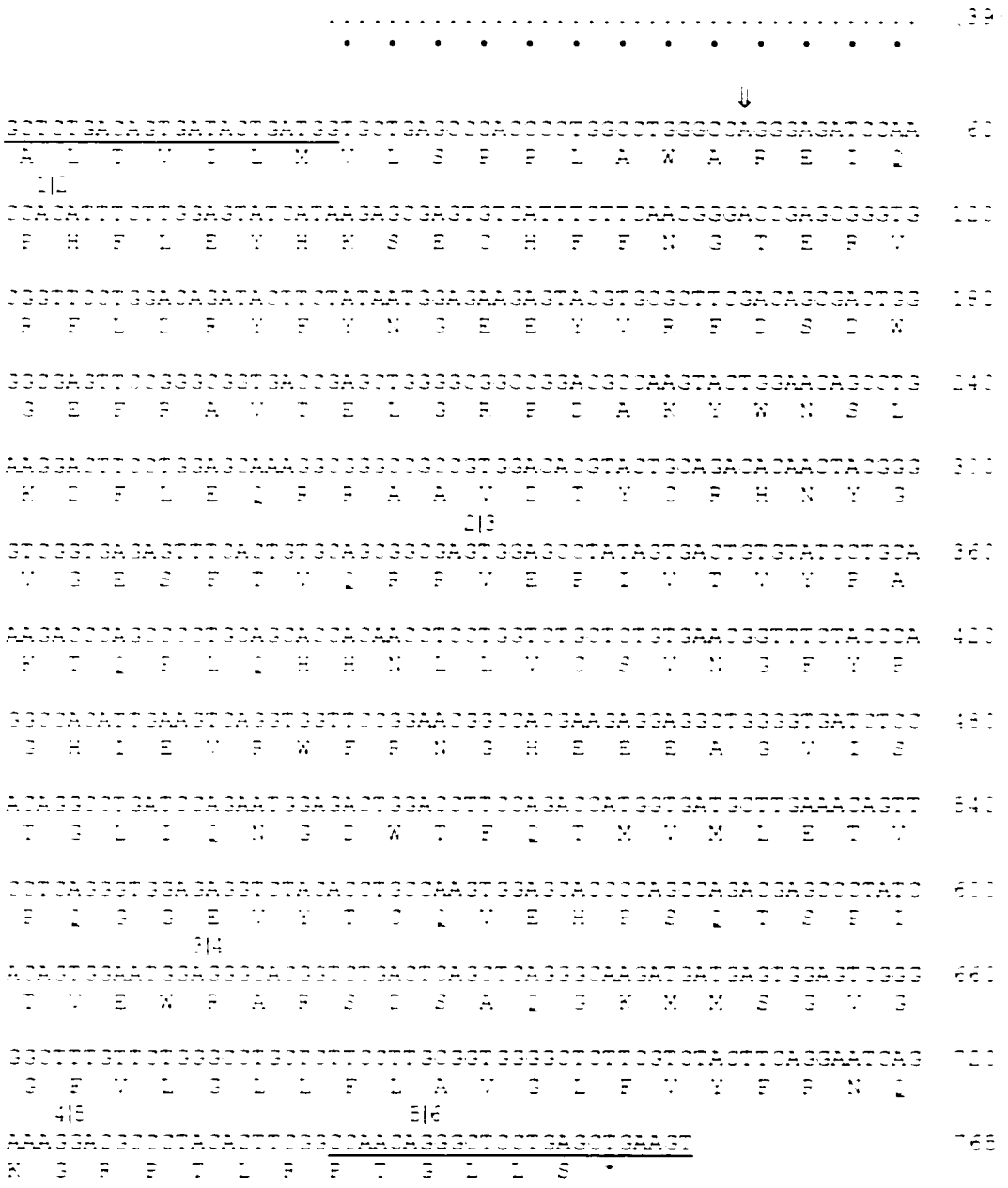
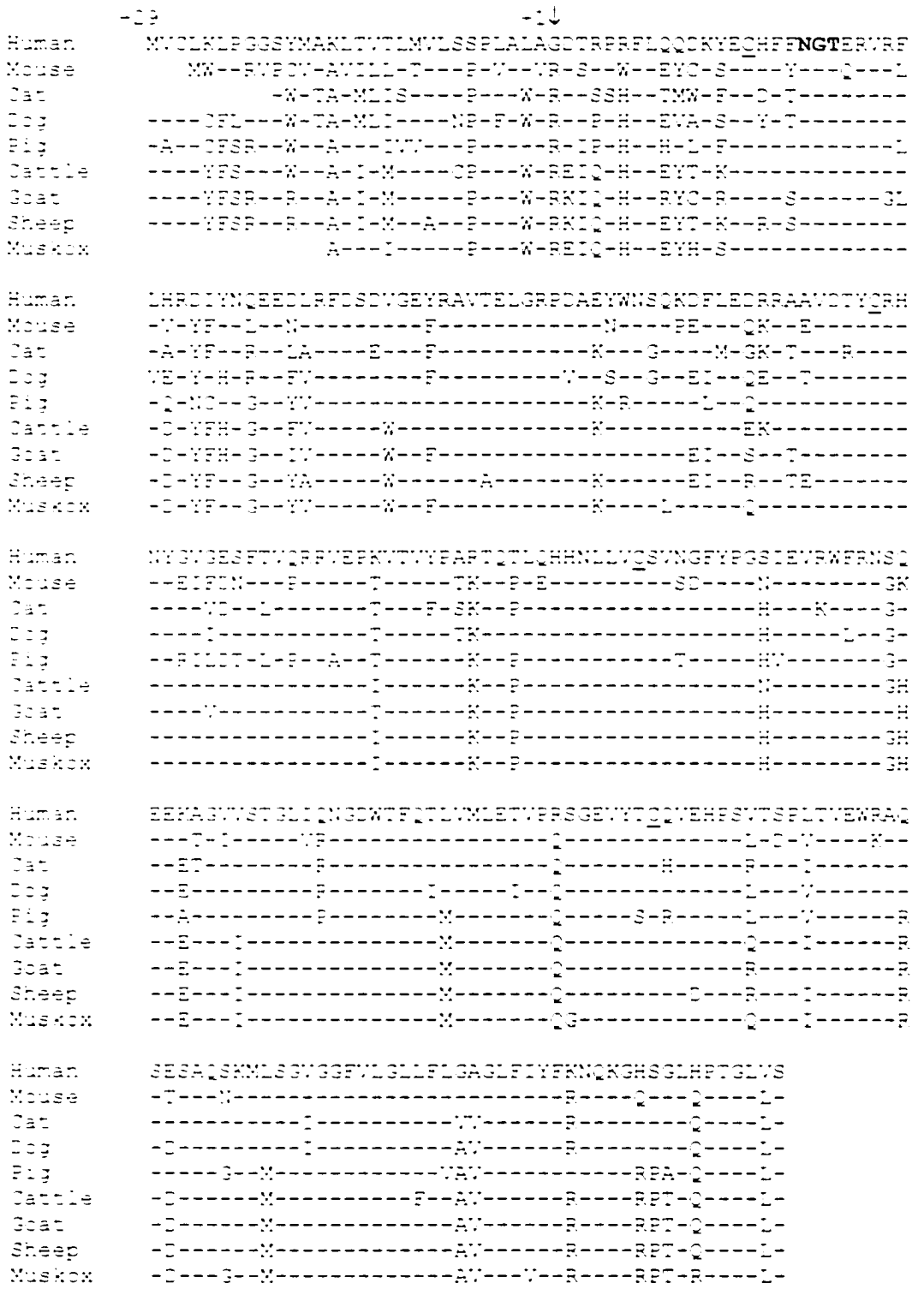


Fig. 2.4 Complete musk ox MHC class II DRB cDNA and its putative amino acid sequence. The signal peptide and the mature DRB border is indicated by a vertical arrow. The borders between exons are marked by vertical lines. Nucleotides used as primers are underlined.

Fig. 2.5 Comparison of the deduced amino acid sequences from the musk ox DRB gene and that of other related species (GenBank accession numbers for humans: M20429; mouse M36939; cat: U51574; Dog: M29611; Pig: M55165; Cattle: D45357; Goat: AB008346; Sheep: 73984). A dash "-" indicates the same amino acid as that of the master sequence (Human). Four conserved cysteine residues were underlined. The potential site of the N-linked glycosylation position is in bold.



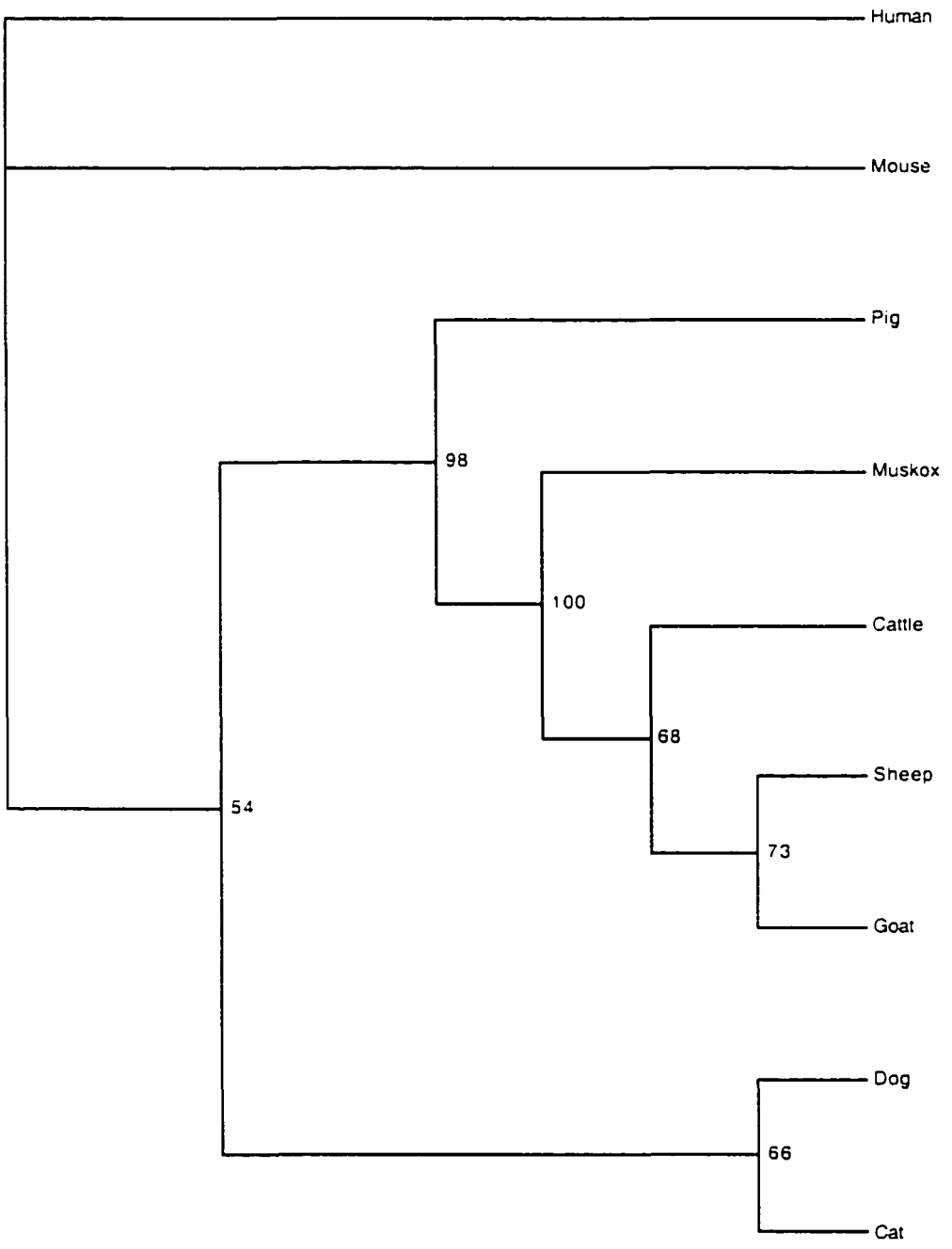
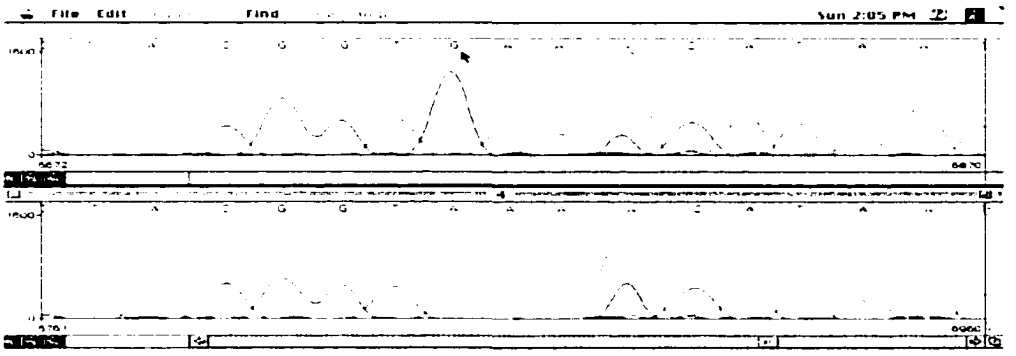


Fig. 2.6 Maximum-parsimony phylogenetic tree of full length DRB sequences. Bootstrap values (1000 replicates) are indicated on the branches. Human DRB sequence was chosen as an outgroup.

```
AATCATGTGATCATCCAAGCTGAGTTCTATCTGAAACCTGAGGAATCAGGC   51
 N  H  V  I  I  Q  A  E  F  Y  L  K  P  E  E  S  G

GAGTATATGTTTGACTTTGATGGTGATGAGATTTTCCACGTGGATATGGAG  102
 E  Y  M  F  D  F  D  G  D  E  I  F  H  V  D  M  E

AAGAAGGAGACGGTGTGGCGGCTTCCAGAATTTGGACGTTTTGCCAGCTTT  153
 K  K  E  T  V  W  R  L  P  E  F  G  R  F  A  S  F

GAGGCTCAGGGTGCCCTGGCCAATATGGCTGTGAACAAAGCCAACCTGGAC  204
 E  A  Q  G  A  L  A  N  M  A  V  N  K  A  N  L  D

ATCATGATGAAGCGCTCCAACAACACCCCAAACACCAATG              244
 I  M  M  K  R  S  N  N  T  P  N  T  N
```

Fig. 2.7 Nucleotide sequence and its deduced amino acid of the monomorphic moose DRA exon 2. Underlined nucleotides are two primers, respectively.



```
DQA(G)  GACCACATTGGCACCTATGGTGTGAACGTCTACCACACATATGGT   45
DQA(C)  ---------------------------------------------
          D  H  I  G  T  Y  G  V  N  V  Y  H  T  Y  G

DQA(G)  CCCTCTGGCCACTATACCCATGAATTTGATGGAGATGAAGAGTTC   90
DQA(C)  ---------------------------------------------
          P  S  G  H  Y  T  H  E  F  D  G  D  E  E  F

DQA(G)  TACGTGGACCTGGAAAAGAGGGAGACCGTCTGGCGTCTGCCTGAG  135
DQA(C)  ---------------------------------------------
          Y  V  D  L  E  K  R  E  T  V  W  R  L  P  E

DQA(G)  TTTAGTAAATTTACAAGTTTTGACCCTCAGGGTGCACTGAGAAAC  180
DQA(C)  ---------------------------------------------
          F  S  K  F  T  S  F  D  P  Q  G  A  L  R  N

DQA(G)  ATAGCTACGGTGAAACATAATTTGGAGATCTTGATTCAAAGGTCC  225
DQA(C)  ----------A----------------------------------
          I  A  T  V  K  H  N  L  E  I  L  I  Q  R  S

DQA(G)  AACTCTACTGCTGCTACCAAC                         246
DQA(C)  --------------------
          N  S  T  A  A  T  N
```

Fig. 2.8 Comparison of musk ox DQA nucleotide sequences between Greenland (G) samples and Coppermine (C) samples. Only one synonymous substitution from G to A has been found in the nucleotide position 192. The DNA sequence profiles indicating the substitution are shown on the top of the page. Underlined regions were used as primers.

```
                  + + +                 + +       ++          +
Ovmo-DQB*01 DFVKQHQFIGQCYFTNGTERVRLVTRYIYNQEEYARFDSDWDEYR
Ovmo-DQB*02 -----F----------------------------------------
Ovmo-DQB*03 ----------------------------------------------
Ovmo-DQB*04 ----------------------------------------------
Ovmo-DQB*05 -----F--K-L----------S-N-----R-------------
Ovmo-DQB*06 -----F----------------------------------------
Ovmo-DQB*07 --------K-L---------------F------FM-----VG---
Ovmo-DQB*08 --------K-V-----------------M--R---------VG---
Ovmo-DQB*09 --------K-V-----------------M--R---------VG---

                    ++  ++   +  + ++  +       +   ++
Ovmo-DQB*01 AVTPLGRPSAEYFNSQEDILEQTRAEVDTVCRNNYQVEAPFTMQRR
Ovmo-DQB*02 ------------W--------RV----------------------
Ovmo-DQB*03 --------------------------------H----D--------
Ovmo-DQB*04 --------D-------------------------------------
Ovmo-DQB*05 ------------W--------R----A-----H----D--------
Ovmo-DQB*06 ----------------------------------------------
Ovmo-DQB*07 -------QP---L--L-GE--RV---------H--L-G--------
Ovmo-DQB*08 ---E--------W------------------H---L-LITSL---
Ovmo-DQB*09 ---E----D---W------------------H---L-LITSL---
```

Fig. 2.9 Alignment of the deduced amino acid sequences of the musk ox MHC DQB (Ovmo-DQB) alleles. A dash "-" indicates that the amino acid is the same as the consensus sequence. A "+" indicates amino acid residues belonging to an antigen-binding site according to the model of Brown et al. (1993).

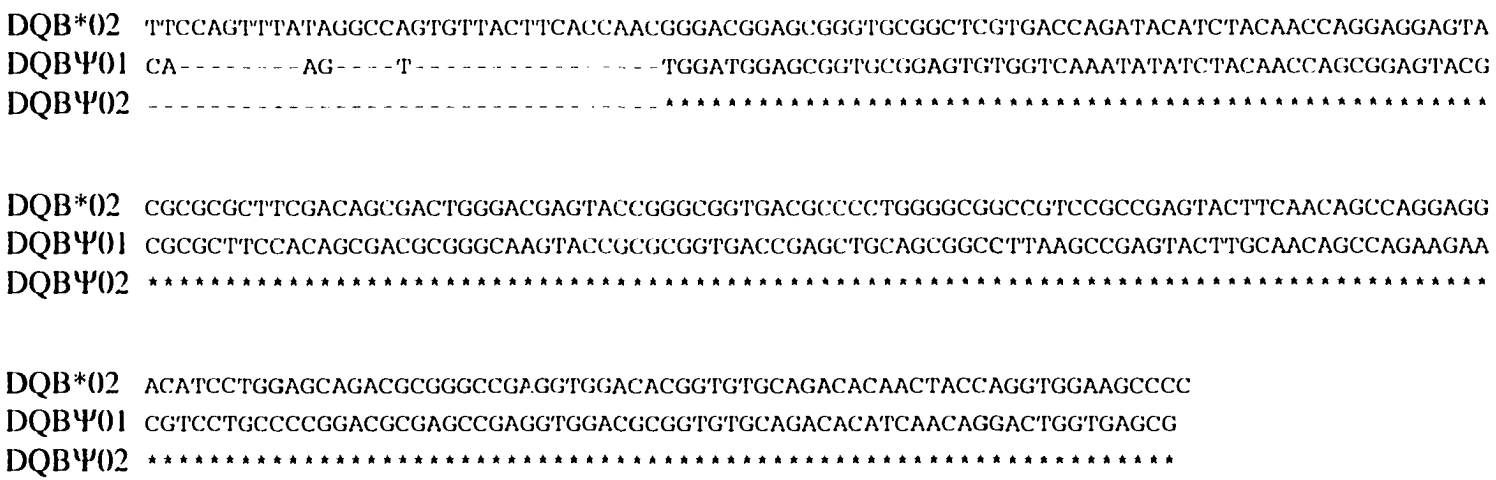


Fig. 2.10 Alignment of musk ox MHC DQB pseudogene sequences. A dash "-" indicates the nucleotide identity with the top sequence (*Ovmo*-DQB*02), an asterisk "*" indicates the nucleotide identity with the second sequence (DQBΨ01).

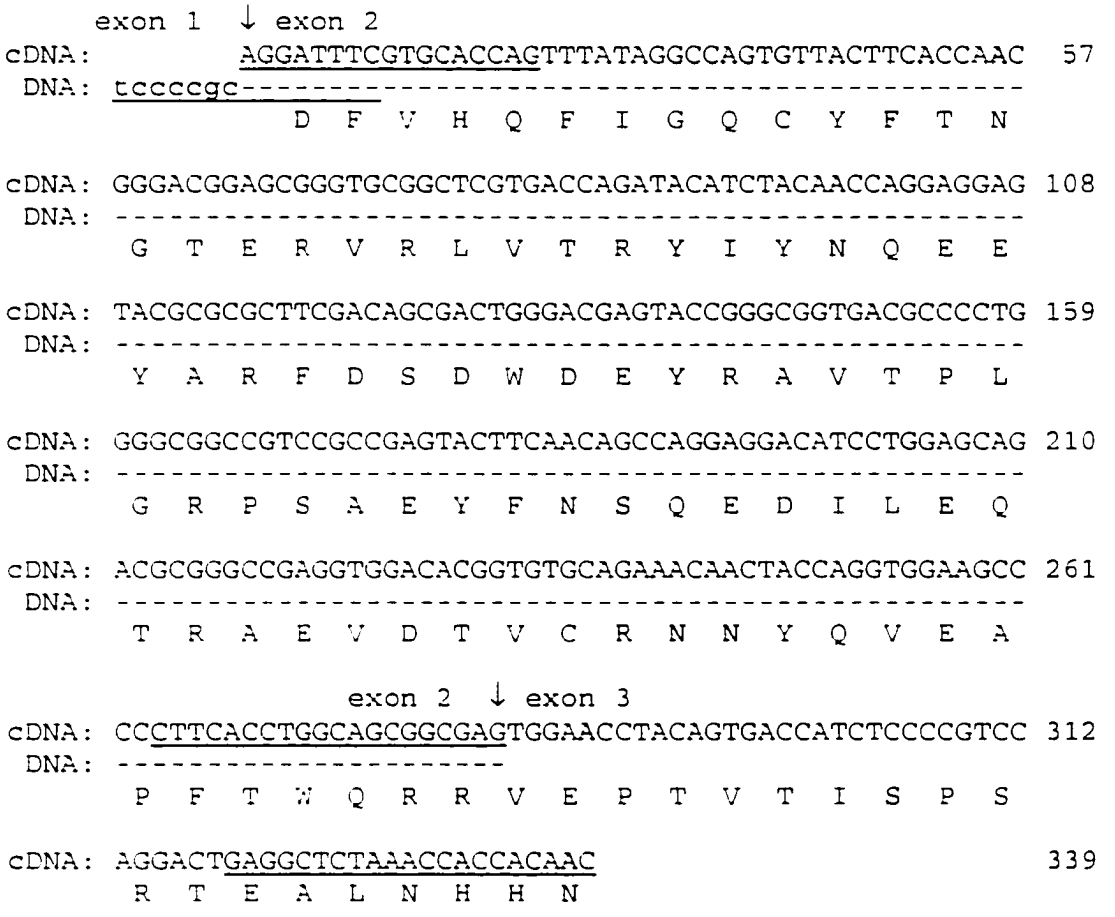


Fig. 2.11 Comparison of genomic and cDNA sequences obtained from the musk ox *Ovmo-* DQB*01 exon 2 region. Nucleotides showed in lower case represent intron sequence. The border between exon 1 and exon 2 is indicated by a vertical arrow. The primers are underlined.

```
DQA*01 GACCACATTGGCACCTATGGTGTGAACGTCTACCACACATATGGT   45
DQA*02 --------------------CA-A--T---------T-G------
         D  H  I  G  T  Y  G  V  N  V  Y  H  T  Y  G
         -  -  -  -  -  -  -  I  -  -  -  -  S  -  -

DQA*01 CCCTCTGGCCACTATACCCATGAATTTGATGGAGATGAAGAGTTC   90
DQA*02 ----------------------------------------------
         P  S  G  H  Y  T  H  E  F  D  G  D  E  E  F
         -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

DQA*01 TACGTGGACCTGGAAAAGAGGGAGACCGTCTGGCGTCTGCCTGAG  135
DQA*02 --------------------A---A--T---------------T-
         Y  V  D  L  E  K  R  E  T  V  W  R  L  P  E
         -  -  -  -  -  -  K  -  -  -  -  -  -  -  V

DQA*01 TTTAGTAAATTTACAAGTTTTGACCCTCAGGGTGCACTGAGAAAC  180
DQA*02 --C---------G--G------------------------------
         F  S  K  F  T  S  F  D  P  Q  G  A  L  R  N
         -  -  -  -  A  G  -  -  -  -  -  -  -  -  -

DQA*01 ATAGCTACGGTGAAACATAATTTGGAGATCTTGATTCAAAGGTCC  225
DQA*02 ------CT-A-----A--GC--------------------A----
         I  A  T  V  K  H  N  L  E  I  L  I  Q  R  S
         -  -  L  M  -  N  A  -  -  -  -  -  -  -  -

DQA*01 AACTCTACTGCTGCTACCAAC                         246
DQA*02 --------------------
         N  S  T  A  A  T  N
         -  -  -  -  -  -  -
```

Fig. 2.12 Comparison of moose DQA nucleotide sequences. DQA*01 is obtained from 20 Alaskan samples, while DQA*02 is obtained from one Russian sample. Moose DQA*01 is identical with musk ox DQA*01 at the nucleotide level. Underlined regions were used as primers.

```
                    + + +                 + +       ++          +
Ala1-DQB*01 DFVKQHQFIGQCYFTNGTERVRLVTRYIYNQEEYARFDSDWDEYR
Ala1-DQB*02 --------K-L---------------F------FM-----VG---
Ala1-DQB*03 --------K-V----------------M--R---------VG---
Ala1-DQB*04 --------K-V----------------M--R---------VG---


                      ++  ++   +  + ++  +       +   ++
Ala1-DQB*01 AVTPLGRPSAEYFNSQEDILEQTRAEVDTVCRNNYQVEAPFTMQRR
Ala1-DQB*02 -------QP---L--L-GE--RV---------H--L-G--------
Ala1-DQB*03 ---E--------W-------------------H-----LITSL---
Ala1-DQB*04 ---E----D---W-------------------H-----LITSL---
```

Fig. 2.13 Alignment of the deduced amino acid sequences of the moose MHC DQB alleles. A dash "-" indicates that the amino acid is the same as the consensus sequence. A "+" indicates amino acid residues belonging to an antigen-binding site according to the model of Brown et al. (1993).

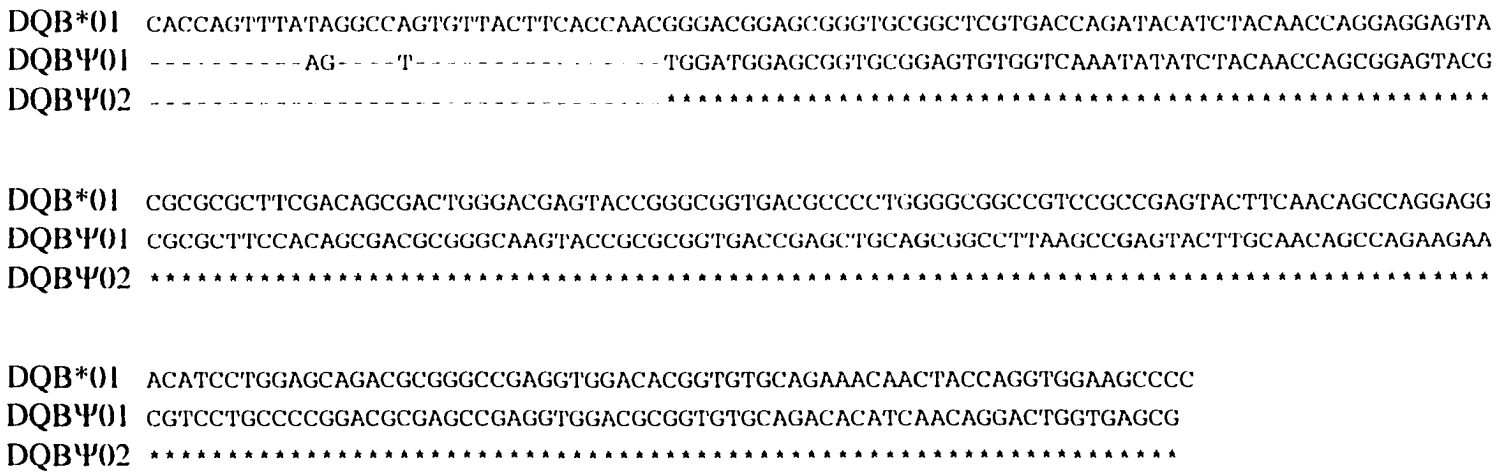


Fig. 2.14 Alignment of moose MHC DQB pseudogene sequences. A dash "-" indicates the nucleotide identity with the top sequence (*Alal*-DQB*01), an asterisk "*" indicates the nucleotide identity with the second sequence (DQBΨ01).

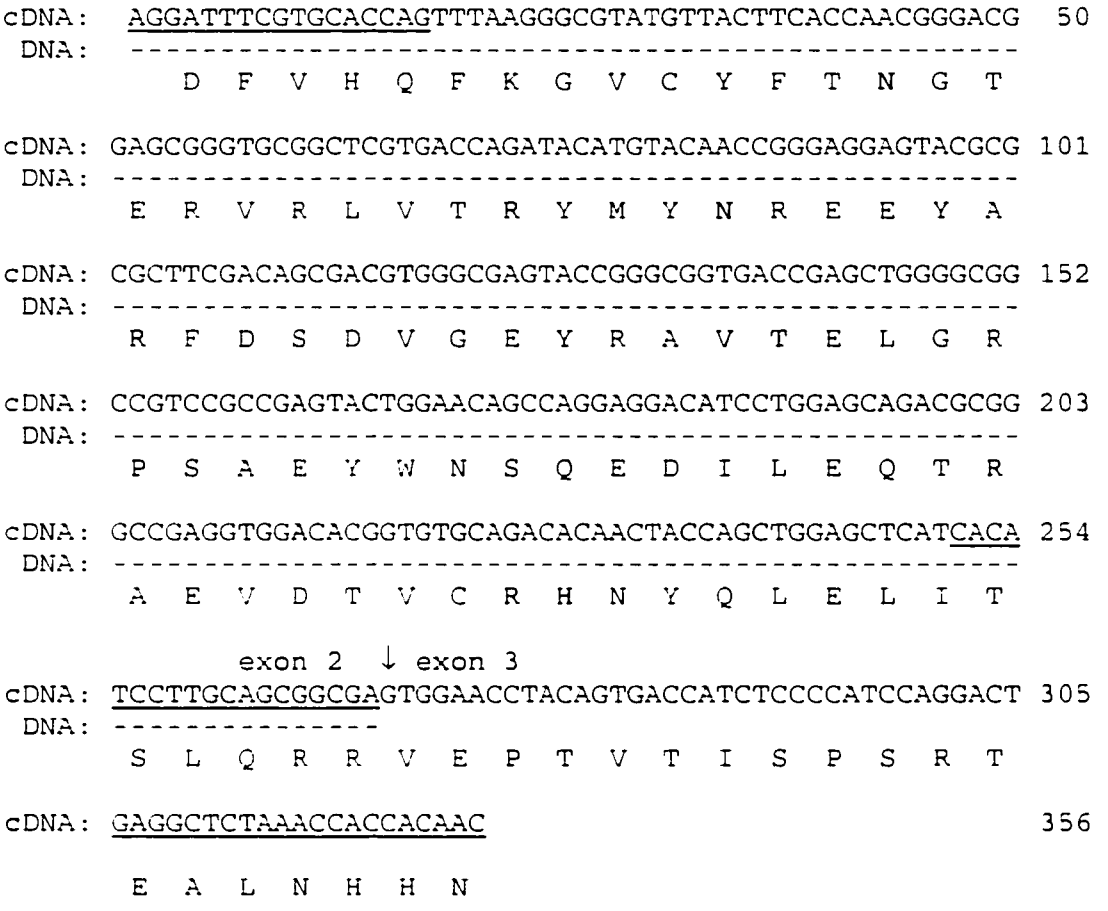


Fig. 2.15 Comparison of genomic DNA and cDNA sequences obtained for the moose *Alal*-DQB*03 exon 2 gene. Nucleotides showed in lower case represent intron sequence. The border between exon 1 and exon 2 is indicated by a vertical arrow. The primers are underlined.

Fig. 2.16 Neighbor-joining phylogenetic tree of DQB exon 2 sequences from musk ox (*Ovibos moschatus*) and moose (*Alces alces*). A cattle DQB sequence (DQB*1001, GenBank accession number U62318) was used as an outgroup to root the tree. Bootstrap percentages of 50 or greater.

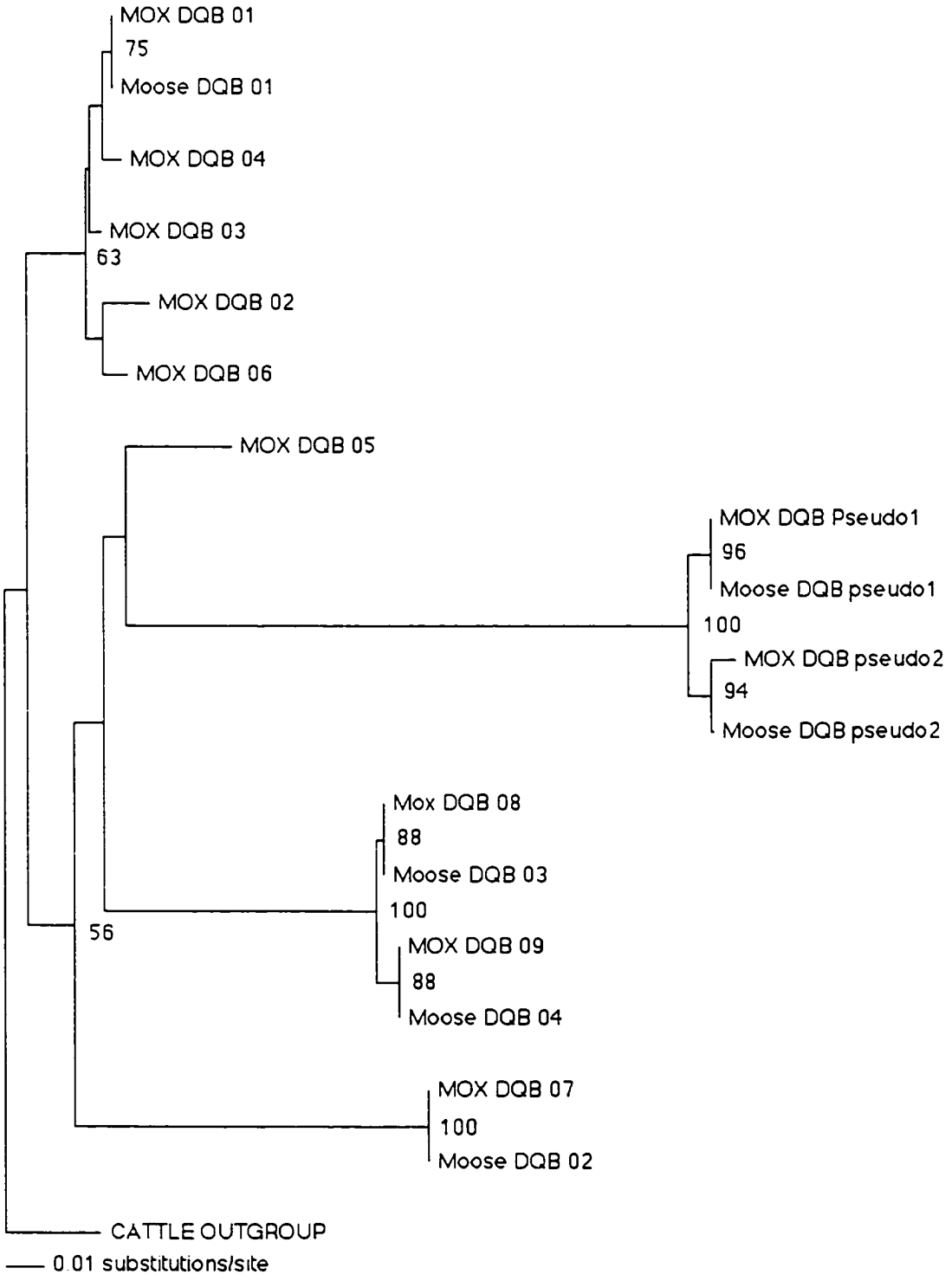


Table 2.1 Summary of primers (started with 5' end) and annealing temperatures used in this study

Genes	Primers for MHC genes	T	References
DRA	DRA-F AATCATGTGATCATCCAAGCTG DRA-R CATTGGTGTTTGGGGTGTTGTTG	55°C	Takada et al. 1998
DRB	DRB1.1 TATCCCGTCTCTGCAGCACATTTC DRB1.2 TCGCCGCTGCACACTGAAACTCTC	60°C	Amills et al. 1995
DQA	DQA-F GACCACATTGGCACCTATG DQA-R1 GTTGGTAGCAGCAGTAGAGTT	60°C	Snibson et al. 1998
DQB	JM05 TCCCCGCAGAGGATTTCGTG JM06 TCCGCCGCTGCCAGGTGAAG MDQBR CTCGCCGCTGCAAGGATGTG	62°C 58°C	van Oorschot et al. 1994, and this study
cDNA1	Primer4 AGCTCTGACAGTGATACTGATGG DRB1.2 TCGCCGCTGCACACTGAAACTCTC	58°C	Swarbrick et al. 1995
cDNA 2	DRBsp TCTGCTGTTCTCCGGCATGGTGTG DRBcy ACTTCAGCTCAGGAGCCCTGTTGG	58°C	Takada et al. 1998
cDNA3	DQBcF AGGATTTCGTGCACCAGTTTA DQBcR2 CACCGAGCAGACCAGCAGGTT DQBcR1 GTTGTGGTGGTTTAGAGCCTC	55°C 58°C	Russell et al. 1997, and this study
OMHC1	F HEX-ATGTGGTGGGCTACAGTCCAT R GCAATGCTTTCTAAATTCTGAGGAA	58°C	Crawford et al. 1995

Note: cDNA1 indicates musk ox DRB exon 2 cDNA; cDNA2 indicates musk ox DRB full length cDNA; cDNA3 indicates musk ox and moose DQB exon 2 cDNA.

Table 2.2 The distribution of 65 musk ox samples based on the synonymous substitution of DQA at the nucleotide position of 192

Sample origin	G	G/A	A
Greenland	25	-	-
Banks Island	8	7	3
Mainland Canada	-	-	22

Table 2.3 Estimated rates of nonsynonymous (dN) and synonymous (dS) substitutions and their ratio in the antigen-binding site (ABS) and non-ABS for the musk ox DQB alleles. Method used for the calculation is based on Nei and Gojobori (1986)

Region	No. of codons	Distances	Musk ox
ABS	16	dN	0.2654±0.0609
		dS	0.0823±0.0576
		dN/dS	3.22 (P=0.06)
Non-ABS	65	dN	0.0405±0.0101
		dS	0.0484±0.0191
		dN/dS	0.84 (P=0.02)
Total	81	dN	0.0744±0.0120
		dS	0.0541±0.0184
		dN/dS	1.38 (P=0.02)

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Chapter III

Genetic diversity at MHC class II DRB locus in reindeer and caribou, *Rangifer tarandus*[2]

Abstract

Objective: To compare the MHC DRB allelic difference between northern species in small populations (musk ox) or solitary lifestyle (moose) and to species with large gregarious populations (reindeer and caribou).

Methods: The polymerase chain reaction (PCR), single-stranded conformation polymorphism (SSCP) analysis, and DNA sequencing techniques were used to assess the genetic variation at MHC class II DRB locus in this study. PAUP and MEGA were used to generate phylogenetic trees and the dN/dS ratio.

Results: Ninety-six samples of reindeer and caribou from different herds in Alaska were examined. Fifteen MHC DRB alleles were identified based on exon 2 sequences. Three of the 15 alleles were reported previously in European populations, while the remaining 12 alleles were found to be unique in North American populations. These alleles showed typical high MHC polymorphism at both nucleotide and amino acid levels. A total of 21 different amino acid substitutions were found, 8 of them were unique to *R. tarandus* with 7 occurring in the putative peptide binding site. Significantly higher rates of nonsynonymous substitutions than that of synonymous substitutions were found among

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[2]Prepared for submission to Immunogentics.

these DRB sequences, indicating the involvement of positive selection in the generation of allelic diversity.

Conclusion: A comparison of amino acid variability among DRB alleles from reindeer and caribou, moose, and white-tailed deer demonstrated that the DRB polymorphism in *R. tarandus* was intermediate. Phylogenetic analysis of cervid DRB alleles indicated that all reindeer and caribou DRB alleles were monophyletic, implying an ancient bottleneck in *R. tarandus*.

Key words: MHC class II DRB, reindeer, caribou, Cervidae, phylogenetic analysis

Data deposition: The sequences reported in this study have been deposited in the GenBank database (accession nos. AF458939-AF458950).

Introduction:

The distribution of reindeer and caribou (*Rangifer tarandus*) is restricted in the northern part of the Holarctic region. Reindeer have a long history of repeated domestication in Eurasia, and they are so called in North America in order to be distinguished from caribou, the native wild populations. There are numerous different populations and subspecies that have been described in this species (Banfield 1961, Bergerud 2000). In Alaska, the current 43,000 reindeer individuals are believed to be descended from around 1,200 reindeer introduced into Alaska from Siberia between 1892 and 1902 (Cronin et al. 1995), and the total number of caribou was recently estimated around 959,000 in 13 different herds (Bergerud 2000). Because there are many different reindeer and caribou populations, it has been of great interest for researchers to characterize the genetic variation among these populations. Genetic markers used include transferrin, microsatellite DNA, mitochondrial D-loop region DNA and some MHC genes (Roed et al. 1986, Olsaker and Roed 1990, Cronin et al. 1995, Wilson et al. 1997, Mikko et al. 1999).

The major histocompatibility complex (MHC) region consists of a cluster of closely linked genes that are involved in the presentation of antigen peptides to the vertebrate immune system (Klein 1986). One of the most characteristic features of MHC genes is the extremely high level of genetic diversity with most of the amino acid substitutions occurring at the peptide-binding site (PBS). Some form of balancing selection has been proposed for the maintenance of MHC polymorphism (Doherty and Zinkernagel 1975, Hughes and Nei 1989, Takahata and Nei 1990). Many studies have

shown that the MHC class II DRB is the most polymorphic locus in artiodactyl species, such as cattle (Sigurdardottir et al. 1991, Mikko et al. 1995b) and sheep (Schwaiger et al. 1994, Paterson et al. 1998). However, levels of DRB variation in some cervid species were found to be from low, such as that found in roe deer (*Capreolus capreolus*; Mikko et al. 1999) and moose (*Alces alces*; Mikko et al. 1995a), to high, such as that found in red deer (*Cervus elaphus*; Swarbrick et al. 1995) and white-tailed deer (*Odocoileus virginianus*; Van Den Bussche et al. 1999). In reindeer and caribou, only 9 DRB alleles were found in European populations (Mikko et al. 1999). An extensive screening of reindeer and caribou DRB alleles in North American populations will provide insight into the tempo and mode of MHC evolution in Cervidae, and also provide information of genetic diversity at MHC for the management of this species.

The objective of this study was to characterize the genetic variation among different reindeer and caribou populations in Alaska focusing on the most variable MHC class II DRB locus. The data were used not only to compare the DRB genetic variation between reindeer and caribou populations in Europe and North America, but also to compare the DRB genetic variation among different northern species with different population size such as musk ox and moose.

Materials and Methods:

Animals:

Included in this study were blood samples from 96 individuals, comprising 58 reindeer specimens and 38 caribou specimens. Samples were collected from different populations across Alaska and Canada (Table 3.1).

SSCP and DNA sequence analysis:

Genomic DNA was extracted from 200 µl of blood sample using Qiaamp blood kit (Qiagen, Chatsworth, California) according to the manufacturer's instructions. Primers (DRB1.1 and DRB1.2) and conditions for polymerase chain reaction (PCR) were used according to Amills et al. (1996) and Mikko et al. (1999). The PCR amplification of DRB exon 2 region was performed with 5 µl of genomic DNA (200-400 ng) as template in a 50 µl reaction containing 5 µl of 10 × buffer, 5 µl of magnesium chloride (12.5 mM), 2 µl of 4 × dNTPs (2.5 mM), 1 µl of each primers (10 pmol), and 0.5 µl of *Taq* Polymerase (2 units). The PCR program consisted of an initial 3 min at 95°C, then a total of 35 cycles of 95°C for 30 s followed by 58°C for 30 s and 72°C for 1 min, and a final 10 min extension at 72°C. All PCR reactions were performed in 96-well plates using GeneAmp PCR System 9700 (PE/Applied Biosystems). PCR products were visualized by running a 5 µl sample on a 2% agarose gel stained with ethidium bromide.

SSCP analysis of DRB was performed essentially according to the method described by Leelayuwat et al. (1994). Briefly, 10-15 µl of PCR product was mixed with 3 µl loading buffer containing denaturing solution (95% formamide, 20 mM EDTA and 0.01% bromophenol blue, pH 8.0), and was heated at 97°C for 5 min, rapidly cooled on crushed ice for 5-10 min and then loaded on a 12.5% non-denaturing polyacrylamide gel

(acrylamide:bis-acrylamide=50:1 in 0.5xTBE). Electrophoresis was performed at 150 V for 50 min at a constant temperature of 20°C. Gels were stained in 0.5 μg ml^{-1} ethidium bromide for 20 min and then visualized on an UV transilluminator. SSCP allelic fragments were isolated from the gel by cutting these fragments from the gel stained by ethidium bromide. Gel fragments were put into 50 μl of sterile water and crushed in a 1.5 ml tube by using a sterile pipette tip. The tube was then put in a 4°C refrigerator overnight. The supernatant was desalted by Qiaquick PCR purification column and 5 μl was used as template in a second round of PCR. The PCR product was used for DNA sequencing after purification. DNA sequencing on both strands was carried out on an ABI 373A instrument using fluorescent dye terminators (Applied Biosystem Inc.).

Phylogenetic analysis:

Distance phylogenetic trees were constructed using the computer program PAUP 4.0 (Swofford. 2000) carrying out a heuristic search performing tree-bisection-reconnection (TBR) branch swapping. The estimation of genetic distances was based on Kimura's two-parameter method (Kimura 1980). Pairwise comparison of nucleotide substitutions between alleles were conducted by the computer program MEGA (Kumar et al. 1993) according to the method of Nei and Gojobori (1986). The ratio between nonsynonymous (dN) and synonymous substitutions (dS) were estimated applying Jukes and Cantor's (1969) correction for multiple hits.

Nomenclature:

The designation of MHC DRB locus in reindeer and caribou follows the method proposed by Klein et al. (1990). Thus the *Rata*-DRB1 was used to name DRB alleles in reindeer and caribou in this study. New alleles detected in this study were named as *Rata*-DRB1*AK01, *Rata*-DRB1*AK02, etc. If the alleles turned out to be the same as that of published, the original name was used (Mikko et al. 1999)

Results:

Allelic fragments from one locus, DRB1, were amplified by PCR from genomic DNA using primers based on caprine DRB exon 2 sequences (Amills et al. 1996, Mikko et al. 1999). SSCP analysis revealed many different banding patterns in all samples tested, indicating heterozygosity for the majority of samples. Based on SSCP analysis and DNA sequencing, 15 unique *Rata*-DRB1 sequences were detected from the 96 samples studied. Every allele was confirmed by the exact sequence being isolated from at least two different individuals. Of the total 15 alleles, 3 of them (*Rata*-DRB1*0102, *Rata*-DRB1*0201, and *Rata*-DRB1*0601) were found to be identical to those reported previously (Mikko et al. 1999), 12 of them (*Rata*-DRB1*AK01 to *Rata*-DRB1*AK12) were new ones that have not been reported before (Fig. 3.1 & Fig. 3.2).

Within these 15 *Rata*-DRB1 allele sequences, 14% (33 out of 236) of nucleotide and 27% (21 out of 78) of amino acid positions showed variation (Fig. 3.1 & Fig. 3.2). Nonsynonymous substitutions (dN) occurred at a significantly higher frequency than that of synonymous substitutions (dS) at the peptide binding site (Table 3.2), which is characteristic of highly polymorphic MHC loci. Thirteen of the 16 (81%) peptide

binding site (PBS) positions had nonsynonymous substitutions while 8 of the 62 (13%) non-PBS positions had nonsynonymous substitutions (Table 3.3). Seven out of 8 unique amino acid residues to Rata-DRB1 were located at the putative PBS (Brown et al. 1993), and extensive sharing of DRB polymorphism was also found at other positions among alleles in Cervidae (Table 3.3). A histogram comparison of amino acid variability among 15 *Rata*-DRB, 10 *Alal*-DRB, and 15 *Odvi*-DRB alleles showed that the distribution and frequency of amino acid substitutions of *Rata*-DRB alleles were between that of *Acal*-DRB and *Odvi*-DRB alleles, indicating that the MHC class II DRB polymorphism in reindeer and caribou was intermediate in Cervidae (Fig. 3.3).

Based on exon 2 sequences, phylogenetic analysis of cervid DRB alleles among reindeer and caribou (this study and Mikko et al. 1999), red deer (Swarbrick et al. 1995), moose (Mikko, et al. 1995), and white-tailed deer (Van Den Bussche et al. 1999) was examined. The tree revealed monophyly of all reindeer and caribou DRB alleles (*Rata*-DRB), similar to that of moose (*Alal*-DRB) (Fig. 3.4). The clade that includes all reindeer and caribou alleles had a sister relationship with one white-tailed deer allele, *Odvi*-DRB12. Other alleles from red deer and white-tailed deer were also found in this major allelic lineage (Fig. 3.4).

Discussion:

This study has revealed that as in European populations, the diversity of the MHC DRB locus in North American populations of *R. tarandus* was intermediate as compared to that of other cervid species such as moose, white-tailed deer and red deer (Fig. 3.3 and

Table 3.3, Van Den Bussche et al. 1999). Fifteen DRB alleles were detected from 96 reindeer and caribou individuals in Alaskan populations. Only 3 of them turned out to be the same alleles as found in European population, implying population differences between these two continents. This result was consistent with the previous transferrin data that 18 alleles were identified in Alaskan populations while 11 alleles were detected in Eurasian populations (Roed and Whitten 1986).

It has been proposed that MHC polymorphism is under parasite-driven selection, that is the coevolution between hosts and parasites acts as a mechanism to maintain the genetic diversity at MHC loci (Doherty and Zinkernagel 1975, Klein and O'hUigin 1994, Parham and Ohta 1996). This mechanism has been recently demonstrated in a large unmanaged population of sheep (*Ovis aries L.*), where MHC allelic variation were correlated well with juvenile survival and parasite resistance (Paterson et al. 1998). In many other species including two cervid species such as red deer and white-tailed deer (Swarbrick et al. 1995, Van Den Bussche et al. 1999), the levels of MHC variation are believed to be maintained by this mechanism.

However, the greatly reduced MHC allelic diversity in moose implies the importance of the environment the host lives in (Mikko et al. 1995a, Ellegren et al. 1996). The intermediate level of DRB diversity in reindeer and caribou as revealed in this study (Fig. 3.3 and Table 3.3) and by Mikko et al. (1999) could be explained as a result of the environmental influence, since reindeer and caribou, like moose, occur in an environment where the diversity of parasites could be low because of the harsh weather. In addition, the likely explanation for the genetic difference between moose and reindeer and caribou

could be their different lifestyles. Moose are solitary and reindeer and caribou are gregarious. It is a common characteristic of gregarious species living in large herds to have extensive polymorphism at MHC loci such as cattle, sheep and red deer, since parasites could be easily transmitted between individual animals and thus increase the selective pressure on MHC genes (Klein and O'hUigin 1994). However, reindeer and caribou live at high latitudes, an environment that is quite different from that of cattle, sheep, and red deer, where the parasite spectrum is supposed to be low. This could be the cause of the intermediate rather than extremely high level of DRB diversity found in reindeer and caribou.

The trans-species polymorphism hypothesis proposed by Klein (1987) explains the phenomenon that many different MHC allelic lineages are conserved through multiple speciation events, and have been found in present-day closely related species. The extreme case of this trans-species polymorphism is that different species could share the same alleles (For class I, Evans et al. 1998, Cooper et al. 1998; for class II, unpublished data from this thesis). Comparison of cervid DRB alleles demonstrates a trans-species persistence of allelic lineages. Extensive sharing of polymorphic amino acid residues in exon 2, especially at the PBS, was found among cervid DRB alleles (Table 3.3). Phylogenetic analysis revealed that many *Ceel*-DRB alleles intermingled in *Odvi*-DRB alleles in several different lineages (Fig. 3.4 and Van Den Bussche et al. 1999). Although all *Rata*-DRB alleles were clustered in one small group, they were still located in a large clade shared by both *Ceel*-DRB and *Odvi*-DRB alleles (Fig. 3.4).

MHC polymorphism can be affected by speciation events, genetic bottlenecks, genetic drift, and selection (Parham and Ohta 1996). It is more likely that ancient genetic bottlenecks could account for the shaping of the contemporary *Rata*-DRB alleles. Firstly, all available *Rata*-DRB alleles found from both European and North American populations were located in one lineage of cervid DRB phylogeny, implying loss of other lineages by genetic bottlenecks during the evolution. Secondly, based on the phylogenetic tree (Fig. 3.4), genetic distances among *Rata*-DRB alleles were greater than that of *Alal*-DRB, indicating that the diversification of *Rata*-DRB alleles was much slower than that of *Alal*-DRB.

Numerous studies have suggested that gene conversion or intraexonic recombination may be a fast mechanism to generate new MHC alleles by reshuffling of polymorphic motifs among some existent alleles (Gyllensten et al. 1991, She et al. 1991, Mikko et al. 1995a, Swarbrick et al. 1995, Parham and Ohta 1996). By examining the alignment of *Rata*-DRB alleles obtained from both this study and data published by Mikko et al. (1999), we found some characteristic patchwork patterns of sequence motifs among some *Rata*-DRB alleles (Fig. 3.2), which fits the model of intraexonic recombination. Despite the growing accumulation of examples showing the possibility of intraexonic recombination in the generation of new alleles, however, another possibility, parallel accumulation of the same mutations or convergent evolution in different allelic lineages, has been argued (Klein and O'hUigin 1995). More investigations of extreme cases, especially those simplified by either natural genetic bottlenecks or man-made genetic bottlenecks, may provide more clear insight into this mechanism by monitoring the events of intraexonic recombination.

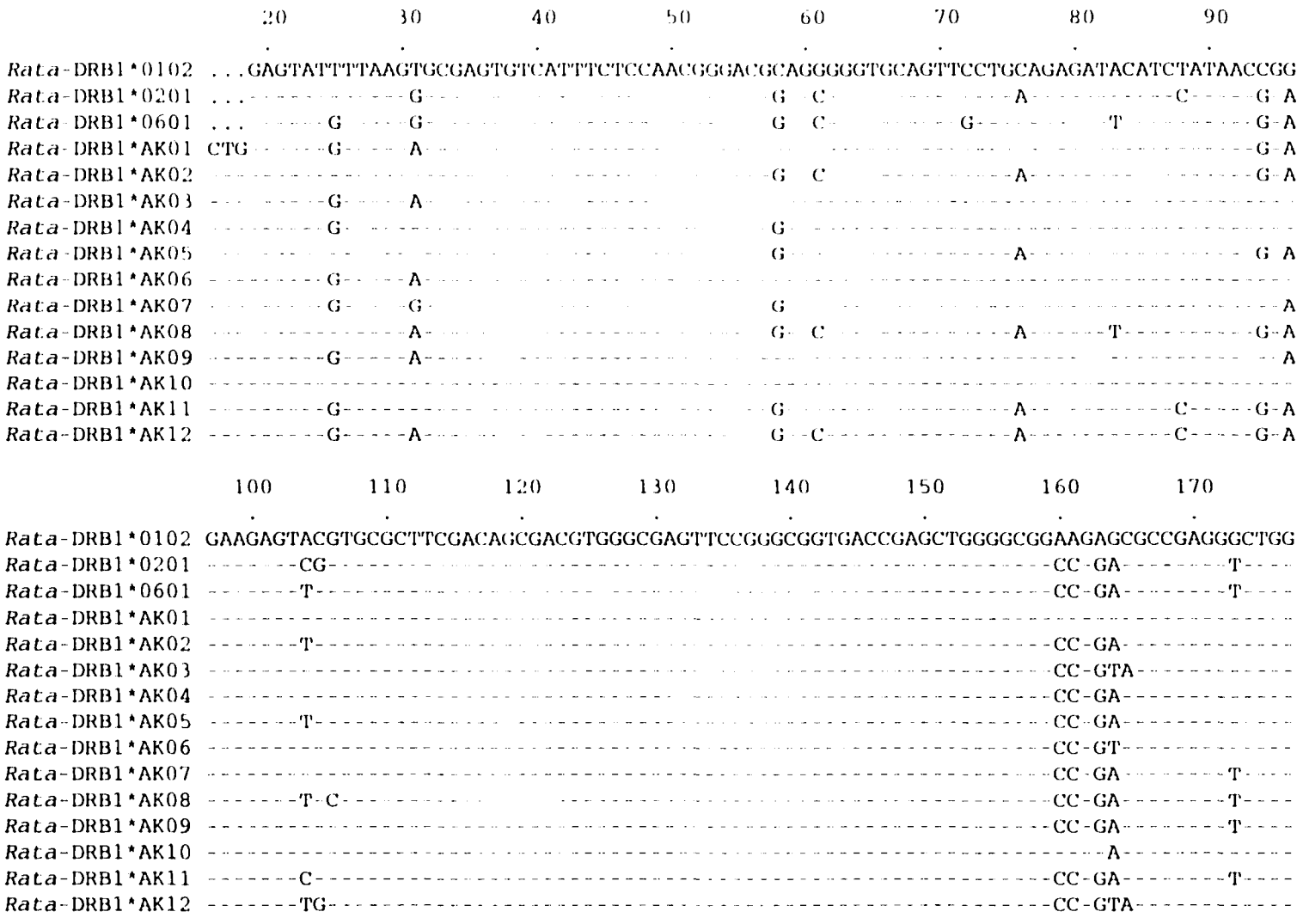

Fig.3.1 Alignment of nucleotide sequences of 15 *Rata*-DRB1 alleles found in this study. A dash (-) indicates identity with the top sequence (*Rata*-DRB1*0102). Numbers indicate the nucleotide positions in the sequences.

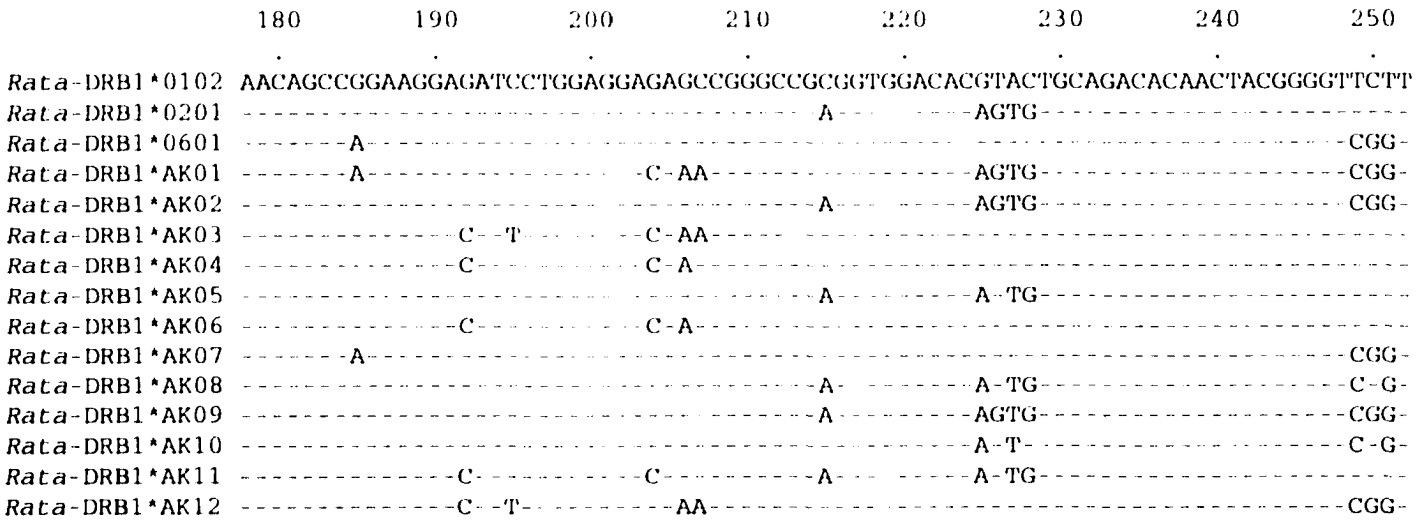

Fig. 3.1 (continued)

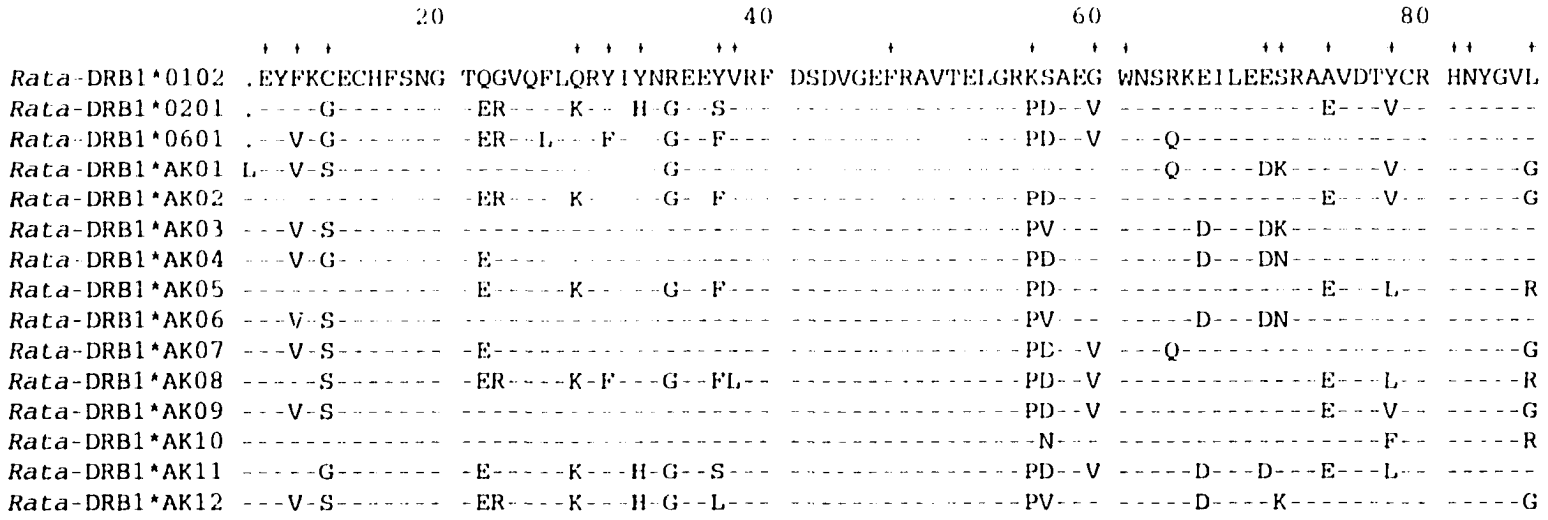

Fig. 3.2 Alignment of the amino acid sequences of the 15 *Rata*-DRB1 alleles found in this study. The amino acid one-letter code was used. A dash (-) indicates identity with the top sequence (*Rata*-DRB1*0102). A cross (+) indicates the amino acid residues forming the putative peptide binding site (Brown et al. 1993). Missing amino acid residues are represented by dots (.).

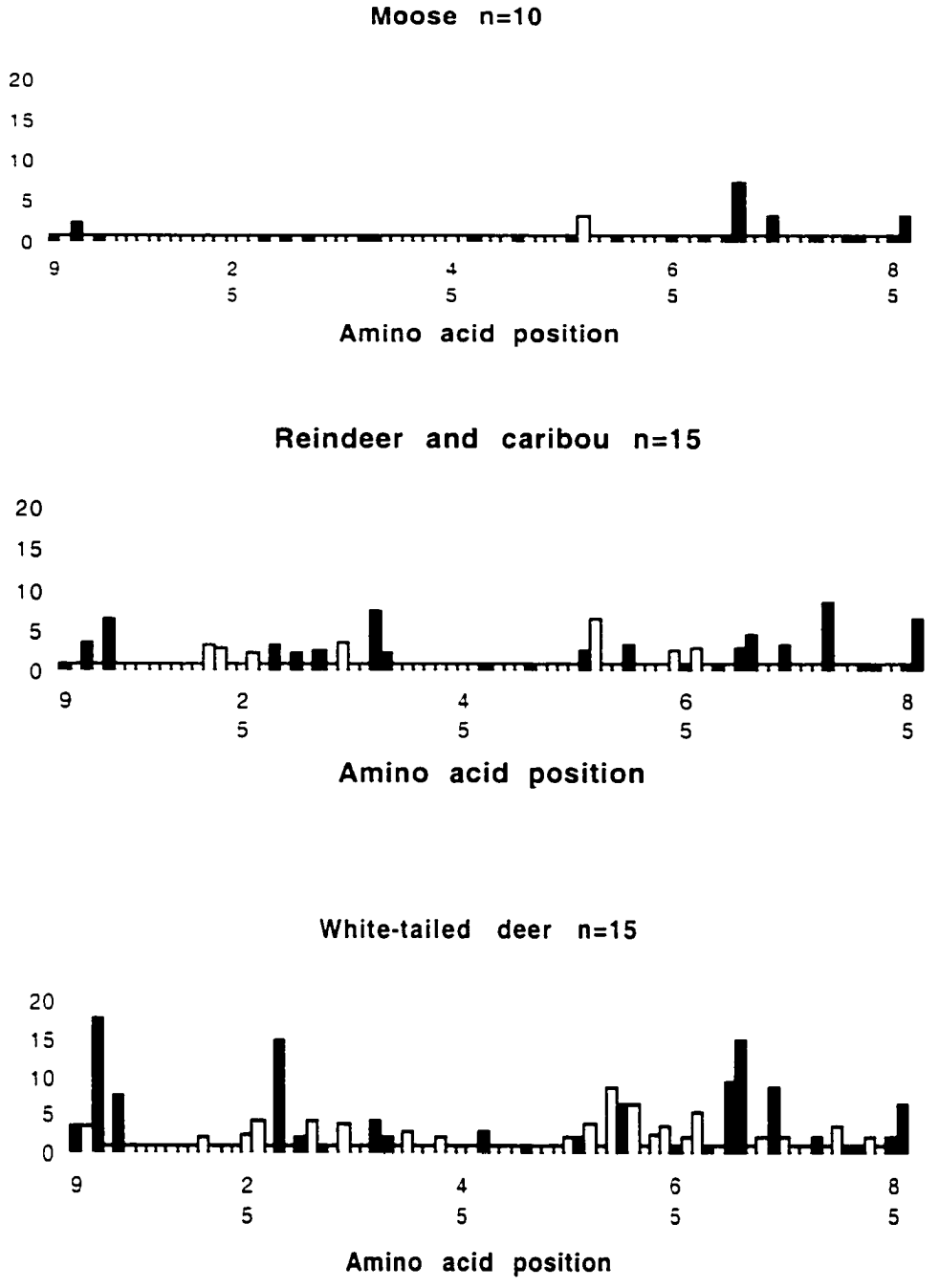

Fig. 3.3 Histogram comparison of amino acid variability among 15 reindeer and caribou (*Rangifer tarandus*), 10 moose (*Alces alces*), and 15 white-tailed deer (*Odocoileus virginianus*) DRB alleles using the method of Wu and Kabat (1970). Black bars represent amino acid residues forming putative PBS (Brown et al. 1993).

Fig. 3.4 Neighbor-joining tree of cervid DRB exon 2 sequences based on 234 bp. *Rata*=reindeer and caribou (*Rangifer tarandus*), *Ceel*=red deer (*Cervus elaphus*), *Odvi*=white-tailed deer (*Odocoileus virginianus*), *Alal*=moose (*Alces alces*). The tree was rooted with a cattle DQB sequence, BLA-DQB*1085 (GenBank accession number: AJ249716). The red deer DRB sequences were taken from Swarbrick et al. (1995), the white-tailed deer sequences were taken from Van Ben Bussche et al. (1999), and the moose sequences were taken from Mikko et al. (1995a).

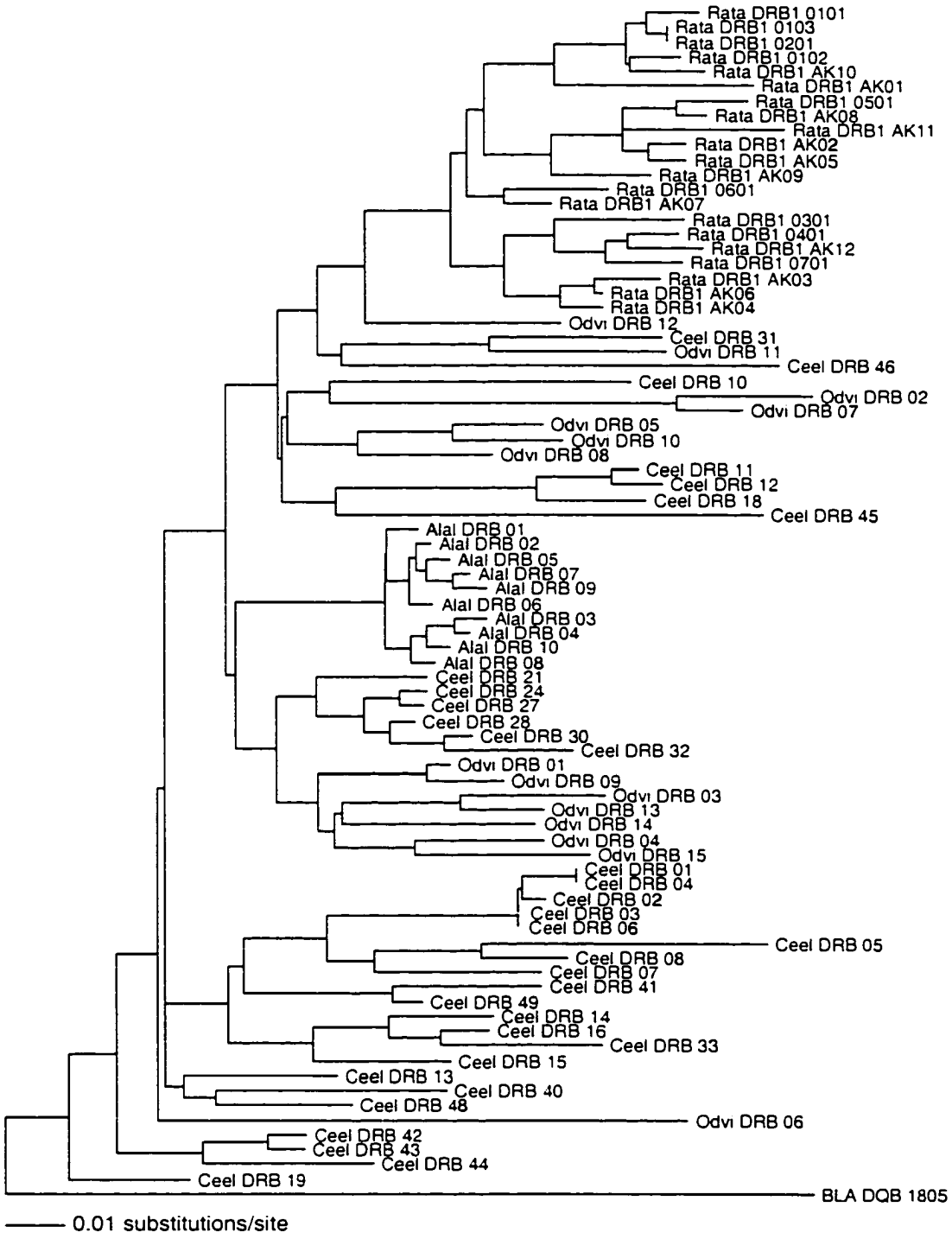

Table 3.1 Number of individuals sampled from different location and herds of *Rangifer tarandus* in this study

Location and Herd	Reindeer	Caribou
LARS*	38	5
Stebbins	5	
Savoonga	5	
Noyakae	5	
Herry	5	
Central Arctic		5
Nelchina		5
Delta		5
North Alaska Peninsula		5
Porcupine		13

*: Large Animal Research Station located in Fairbanks, Alaska

Table 3.2 Estimated rates of nonsynonymous (dN) and synonymous (dS) substitutions and their ratio, where N is the number of codons in the DRB exon 2 tested and P is the probability of significance between dN and dS

Positions	N	dN	dS	dN/dS	P
Antigen binding	16	0.2017 ± 0.0429	0.0571 ± 0.0477	3.53	<0.001
Non-antigen binding	62	0.0398 ± 0.0092	0.0350 ± 0.0183	1.14	<0.02
All	78	0.0713 ± 0.0106	0.0388 ± 0.0165	1.84	<0.005

Table 3.3 Summary of polymorphic amino acid substitutions at exon 2 of the DRB alleles in Cervidae. The standard one-letter amino acid code was used. An asterisk (*) indicates peptide binding site residues (Brown et al. 1993). Data other than reindeer and caribou were based on Van Den Bussche et al. (1999)

Codon Position	Reindeer Caribou	Moose	Roe deer	Red deer	White-tailed deer
9*	E	E	M	EML	ESV
10	Y	Y	Y	HQY	HY
11*	FV	AH	T	AFHLPSTY	AFGHPV
12	K	K	T	KT	K
13*	CGS	S	G	AGKRS	AGKS
16	H	H	H	HPY	H
18	S	F	S	FPS	S
22	EQ	E	E	DEQ	EQ
24	V	V	V	MV	V
25	Q	R	R	EGQR	QR
26	FL	F	F	FLSY	FLY
28*	KQ	D	D	ADEGQ	DEFQV
30*	FY	Y	Y	Y	DY
31	I	I	F	FIV	FIV
32*	HY	Y	Y	CHY	Y
33	N	N	N	KNS	N
34	GR	R	G	EGRW	GKQR
36	E	E	E	EG	E
37*	FSY	Y	FY	FTY	FIY
38*	LV	V	V	ALV	LV
39	R	R	RS	R	R
40	F	F	F	FY	FY
42	S	S	S	CGS	S
43	D	D	D	D	DN
44	V	V	W	VW	V
46	E	E	E	EQ	E
47*	F	Y	Y	FY	FY
48	R	R	R	QR	R
52	E	E	E	EK	E
55	R	R	Y	LR	QR
56*	KP	P	P	PR	PT
57	DNSV	DS	DLS	DSV	DES
59	E	K	EK	EK	DEK
60*	GV	Y	GY	GHLY	DGNY
61*	W	W	W	LWY	FWY
63	S	S	S	RS	RS
64	QR	Q	Q	LQR	QR

Table 3.3 (continued)

Codon Position	Reindeer Caribou	Moose	Roe deer	Red deer	White-tailed deer
65	K	E	K	KR	K
66	DE	D	E	DE	DE
67	I	I	IF	FILY	FIL
68	L	L	L	LM	L
70*	DE	Q	QR	DEQR	DEQRY
71*	KNS	GRT	GR	AEHKLNRST	KLNRSW
72	R	R	R	GR	R
73	A	A	A	AG	AT
74*	AE	AE	AE	AEN	AFIL
75	V	V	V	V	AV
76	D	D	D	DN	D
77	T	T	T	RT	T
78*	FLVY	Y	Y	FVY	VY
80	R	R	R	R	IRS
81*	H	H	H	HY	H
82*	N	N	N	DN	N

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Chapter IV

Extensive MHC class II DRB diversity in polar bear *Ursus maritimus*: Four identical alleles were found sharing with dog[3]

Abstract

Objective: To examine the difference of the major histocompatibility complex (MHC) variation between herbivores and carnivores in the Arctic, we characterized genetic diversity at the most polymorphic class of MHC genes (class II DRB locus) in polar bears.

Methods: DRB exon 2 was amplified by PCR. The PCR products were cloned and sequenced. Phylogenetic relationship among DRB alleles were analyzed using PAUP program.

Results: A total of 25 DRB alleles were detected in 44 samples collected from both Siberia and Canada. Most of the amino acid substitutions occurred at positions within the peptide binding site, and the rate of nonsynonymous substitution was significantly higher than that of the synonymous substitution, indicating the involvement of positive selection. The general pattern of polar bear DRB was extremely similar to that of canine DRB1, both contained three highly variable regions. Phylogenetic analysis showed that the most polar bear DRB alleles intermingled with canine DRB1 alleles, and four alleles appeared to be identical at the nucleotide level between these two different species which

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[3]Prepared for submission to Immunogenetics.

most likely split 10 to 15 million years ago.

Conclusion: Extensive genetic diversity at the MHC DRB locus was detected in polar bears, indicating the difference of MHC variability between herbivores and carnivores in the Arctic. Trans-species polymorphism at DRB locus was found between two distantly related species, polar bear and dog.

Key words: polar bear, MHC class II DRB, trans-species polymorphism, phylogenetic tree

Data deposition: The sequences reported in this study have been deposited in GenBank database (accession nos. AF458914-AF458938).

Introduction:

Polar bears (*Ursus maritimus*) are large mammals that live on ice-covered sea regions throughout the circumpolar Arctic (Paetkau et al. 1995). As a typical Arctic species, polar bears are almost exclusively carnivorous and are well adapted to exploit the Arctic marine environment. Their main food source is seals (Smith 1980). Nineteen distinctive populations are currently recognized (Paetkau et al. 1999). Due to their low reproductive rates and low densities, polar bears were thought to be vulnerable to environmental changes and over-hunting (Taylor et al. 1987, Stirling and Delocher 1993). A low level of genetic variation was found in polar bears based on the data from allozymes and mitochondrial (mt) DNA (Larsen et al. 1983 and Cronin et al. 1991). Recently, microsatellite DNA analysis showed that there was considerable genetic variation detected in polar bear populations (Paetkau et al. 1995 and 1999). The microsatellite DNA data indicated that gene flow between local polar bear populations is restricted despite the long-distance movements undertaken by polar bears.

MHC gene products are glycoproteins that are expressed on the cell surface as heterodimers. The basic function of MHC molecules is for immunological recognition; that is, to bind and present antigenic peptides to T cells, and initiate immune responses to the invading parasites. This function of MHC molecules implies that genetic variability at the MHC loci may be strongly influenced by the spectrum of parasites the host encounters (parasite-driven hypothesis) (Klein and O'hUigin 1994, Paterson et al. 1998). Some form of balancing selection, most likely overdominant selection (a heterozygote has a higher selective advantage than either homozygote) and frequency-dependent

selection (rare alleles have a higher selective advantage), have been proposed to explain the maintenance of this genetic variability (Hughes and Nei 1988 & 1989, Klein et al. 1993).

MHC genes are among the most variable ones so far found in any vertebrate. Enormous genetic diversity has been documented at MHC class II DRB gene exon 2 region in many mammals studied so far (e.g. cattle, deer, cat, and dog) (Andersson et al. 1991, Swarbrick et al. 1995, Yuhki et al. 1997 and Wagner, et al. 1996). However, studies of some species, such as African cheetah, moose, musk oxen and European beaver, have demonstrated either a low level of MHC DRB diversity or even monomorphism at this locus (O'Brien et al. 1985, Mikko et al. 1995 and 1999, and Ellegren et al. 1993).

MHC polymorphism can be affected by several factors, such as population divergence, genetic bottlenecks, selective pressure, and genetic drift (Parham and Ohta 1996). An environment with low levels of parasite diversity, such as a marine environment and/or the Arctic, is correlated with low levels of MHC allelic diversity (Slade 1992, Murray et al. 1995, Mikko et al. 1995 & 1999). For the Arctic region, the herbivores moose and musk oxen were found to exhibit either very low variation or monomorphism at the DRB locus. In an attempt to examine the difference of MHC variation between herbivores and carnivores in the Arctic, we characterized genetic diversity at the class II DRB locus in polar bears in this study.

Materials and Methods:

Animals:

Polar bear blood and tissue samples of from both North Canada and Siberia were used in this study. In addition, several samples of brown bear (*Ursus arctors*) and black bear (*Ursus americanus*) were also included. For polar bears, 16 individuals from Siberia and 28 individuals from Canada were examined. For brown bears, two of Alaskan coastal samples and one of ABC (Admiralty, Baranof, and Chichagof in southeast Alaska) islands sample as well as one European sample were used. For black bears, 5 samples from Alaska were included.

PCR, cloning and sequencing:

Genomic DNA was extracted from 200 µl of blood sample using the QiaAmp blood kit (Qiagen, Chatsworth, California) according to the manufacturer's instructions. The polar bear DRB exon 2 region was amplified by two PCR performances (Fig. 4.1). The first PCR was carried out using primers based on dog DRBB1 nucleotide sequence (Kennedy et al. 1998). The PCR amplification was performed with 5 µl of genomic DNA (200-400 ng) as template in a 50 µl reaction containing 5 µl of 10 × buffer, 5 µl of magnesium chloride (12.5 mM), 2 µl of 4 × dNTPs (2.5 mM), 1 µl of each primers (10 pmol), and 0.5 µl of *Taq* Polymerase (2 units). The PCR program consisted of an initial 3 min at 95°C, then a total of 35 cycles of 95°C for 30 s followed by 55°C for 30 s 72°C for 1 min, and a final 10 min extension at 72°C. The amplified band of expected size was cut from the agarose gel, purified using a gel extraction kit (Qiagen, Chatsworth, California), and used as the template for the second semi-nested PCR reaction. The second semi-nested PCR was conducted using the dog DRB forward primer (Wagner et

al. 1996) and the cattle DRB reverse primer (Amills et al. 1996). The second PCR amplification was performed with 2 µl of the purified first PCR product DNA (20 ng) as template in a 50 µl reaction containing 5 µl of 10 × buffer, 2 µl of 4 × dNTPs (2.5 mM), 1 µl of each primers (10 pmol), and 0.5 µl of *Taq* Polymerase (2 units). The PCR program consisted of an initial 3 min at 95°C, then a total of 35 cycles of 95°C for 30 s followed by 58°C for 30 s 72°C for 1 min, and a final 10 min extension at 72°C. The second PCR products were also cut from gel, purified using the gel extraction kit (Qiagen, Chatsworth, Calif.) and cloned into pT7Blue-3 using Perfectly Blunt Cloning Kits (Novagen, Madison, Wisconsin). Plasmid DNA was purified from white clones using Wizard Plus Minipreps (Promega, Madison, Wisconsin). An average of 6-10 clones were selected and sequenced for each sample. DNA sequencing on both strands was carried out on an ABI 373A instrument using fluorescent dye terminators (Applied Biosystem Inc.).

Phylogenetic analysis:

A neighbor-joining phylogenetic tree was constructed using the computer program PAUP 4.0 (Swofford, 2000) carrying out a heuristic search performing tree-bisection-reconnection (TBR) branch swapping. Estimation of genetic distances was based on Kimura's two-parameter method (Kimura 1980). Pairwise comparison of nucleotide substitutions between alleles were conducted using the computer program MEGA (Kumar et al. 1993) according to the method of Nei and Gojobori (1986). The ratio between nonsynonymous (dN) and synonymous substitutions (dS) were estimated applying Jukes and Cantor's (1969) correction for multiple hits.

Nomenclature:

The designation of MHC DRB locus in polar bears follows the method proposed by Klein et al. (1990). *Urma*-DRB thus was the nomenclature used to name DRB alleles in polar bears in this study. New alleles detected in this study were named as *Urma*-DRB01, *Urma*-DRB02, etc.

Results:

A total of 25 *Urma*-DRB alleles were found in all 44 samples tested in this study. The DRB polymorphism showed some characteristic features of a highly polymorphic MHC locus (Fig. 4.2 & 3). First, the number of observed alleles was very large. Second, the genetic distance between alleles was large with 2-15 amino acid substitutions out of 81 residues, and 28% of the amino acid positions were polymorphic. Third, most amino acid substitutions occurred at positions that form the putative peptide binding site (Fig. 4.3 and Brown et al. 1993). Fourthly, the frequency of nonsynonymous substitutions was significantly higher than that of synonymous substitutions in positions that form the putative peptide-binding site (Table 4.1), indicating that this locus has been exposed to positive selection.

The general pattern of *Urma*-DRB was extremely similar to that of canine DRB1, both included three highly polymorphic regions (Fig. 4.2 & 3). Extensive sharing of DRB polymorphism was observed when comparing the 25 *Urma*-DRB alleles obtained from this study with the 54 canine DLA-DRB1 alleles described by Kennedy et al. (2001). In addition, some patchwork patterns were found in the three highly variable

regions of these DRB alleles, implying the involvement of gene conversion. One of the most frequent alleles, *Urma*-DRB22, was found among polar bear, brown bear, and black bear (data not shown). Moreover, four *Urma*-DRB alleles were found to be identical to the corresponding canine alleles. These shared alleles were *Urma*-DRB22/DDRB1*00601, *Urma*-DRB23/DDRB1*01201, *Urma*-DRB24/DDRB1*01502, and *Urma*-DRB25/DDRB1*00201 (Fig. 4.4).

Fig. 4.4 shows a neighbor-joining phylogenetic tree with the 25 *Urma*-DRB alleles obtained in this study and the 54 canine DLA-DRB1 alleles (Kennedy et al. 2001 and personal communication). There were several major allelic groups in this tree. All *Urma*-DRB alleles were intermingled with the majority of canine DLA-DRB1 alleles in most of those groups except one in the middle. No *Urma*-DRB allele was found in this group which contained only 23 DLA-DRB1 alleles including alleles between DDRB1*00101 and DDRB1*02501 (Fig. 4.4). This result correlated well with the allelic alignment comparison (data not shown), implying that polar bear may have lost alleles in this allelic lineage during the evolution.

Discussion:

We characterized the genetic diversity at the MHC class II DRB locus in polar bears (*U. maritimus*) in this study. Contrary to the previously reported low levels of genetic variation at some different genetic markers such as allozyme and mitochondrial DNA (Larsen et al. 1983 and Cronin et al. 1991), this study revealed that there was extensive genetic diversity at the MHC DRB locus. A total of 25 *Urma*-DRB alleles

were detected in the 44 samples tested. In addition to the very similar general DRB patterns between polar bear and dog, four *Urma*-DRB alleles turned out to be identical to the corresponding canine DRB1 alleles at nucleotide level, indicating trans-species polymorphism in these two distantly related species which split at least 5 million years ago and probably 10 to 15 million years ago. Currently, 14 of 19 distinctive polar bear populations are located in North America (Paetkau et al. 1999, Pasitschniak-Arts and Messier 2000). Samples included in this study were collected randomly from both Siberia and North Canada, representing at least 2 of the 19 different populations.

A crucial question is whether the allelic identities between polar bear and dog are real or due to contamination. The latter possibility seems unlikely. First, the first PCR amplification of polar bear DRB exon 2, although using canine DRB primers, showed multiple bands on the agarose gel (Fig. 4.1) which is quite different from that of dog's one distinctive band, indicating different starting sample genomic DNA. Second, we started the amplification of polar bear DRB before any DRB amplification from other species, making it unlikely to get contamination. Third, the only two dog DRB alleles detected by me in our laboratory were limited to the only two alleles found in Doberman pinschers, DDRB*00601 and DDRB*01201, which is quite different from the 25 DRB alleles detected in polar bears. Finally, the result was confirmed by repeats of independent amplifications both in the same laboratory and in another independent laboratory.

Unlike the two northern herbivorous species such as moose and musk oxen, whose MHC DRB variability were either very low or even monomorphic (Mikko et al. 1995 & 1999), carnivorous polar bears, although living in the Arctic and in low density, showed

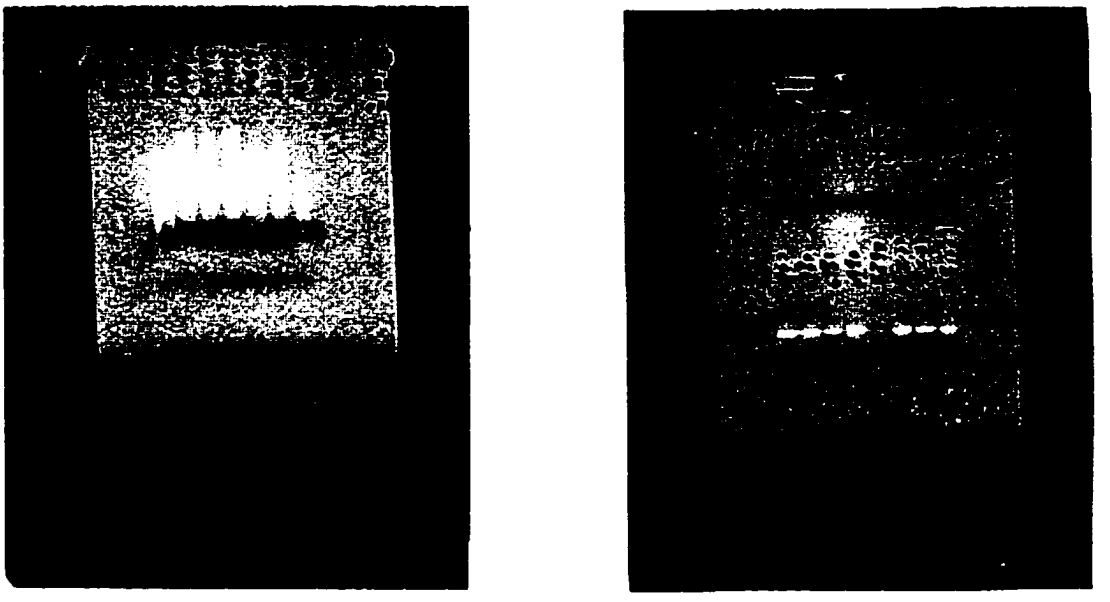
extremely high level of DRB diversity. Given the important role of MHC molecules in the host defense system against infectious diseases, it is plausible, according to the parasite-driven selection hypothesis (Klein and O'hUigin 1994), that the high DRB variability in polar bears may be linked with their food sources. Polar bears are almost exclusively carnivorous, feeding on seals and fish. The food source may transmit pathogens to polar bears and thus increase the selective pressure on the MHC alleles.

An alternative explanation could be due to the persistence of MHC diversity from ancestors. We suspect the ancestors of polar bears had high MHC diversity because they were omnivorous. Polar bear and brown bear have been suggested as sister taxa from ancestral brown bear based on fossil data and genetic data (Kurten 1964, Kowalska 1965, Shields and Kocher 1991). It has been estimated that polar bears may be derived from coastal brown bears, most likely somewhere in Siberia, about 300,000--400,000 years ago (Talbot and Shields 1996) when higher genetic diversity has been detected in brown bears based on ancient mitochondrial (mt) DNA sequences (Leonard et al. 2000). If so, the extensive MHC variability in polar bears could be inherited from brown bears, a species that can exploit a variety of animal and plant foods in much wider geographic ranges. Since the mutation rate at MHC loci is extremely low (Klein et al. 1993), a period of 300,000--400,000 years is not sufficient for the accumulation of mutations to generate the extensive MHC variability found in polar bears from this study.

One of the characteristics of MHC genes is the retention of allelic lineages in different species. Klein (1987) proposed trans-species polymorphism to explain this phenomenon that the contemporary species inherit much of their MHC polymorphism from their common ancestral species through multiple speciation events. A prediction of

this hypothesis is that allelic sharing could be found in related species. An extreme case of this prediction is that two species share one identical allele. To date, two similar results have been published independently that a MHC class I allele is shared by two different primate species which diverged 0.7 and 2.3 million years ago, respectively (Evans et al. 1998, Cooper et al. 1998). The finding of four identical DRB alleles shared by polar bears and dogs in this study provided the first example of trans-species polymorphism at MHC class II DRB locus (Fig. 4.4). This result allows us to estimate that the retention of MHC DRB alleles in both polar bears and dogs may start from 10 to 15 million years ago when these two species diverge.

Although the exact mechanism for this strict trans-species polymorphism is not clear so far, some factors that might influence the MHC mutation rate have been suggested. These factors include the number of alleles at a particular locus, the genetic variability of alleles, population size, genetic drift, and selective pressures (Parham and Ohta 1996, Evans et al. 1998). The most frequent allele in polar bear, *Urma*-DRB22, has also been found in dogs and wolves as one of the most common alleles that was designated as DDRB1*00601 (Kennedy et al. 1998 & 2001, Hedrick et al. 2000). Considering the fact that both polar bear and dog are carnivorous and their ancestors (brown bear and gray wolf) share a similar geographic distribution, it is quite likely that the shared alleles may be responsible for a common array of antigens that these two species have been facing. In fact, some studies have shown common parasites these two species sharing, such as *Trichinella* (Forbes 2000). An antigen-binding assay of the shared allele could provide further information of the array of antigens.



A B

Fig. 4.1 PCR amplification of polar bear DRB (*Urma*-DRB) exon 2 region. The first PCR products are shown in photo A. The expected size band (the third one from the top) was cut from the gel, purified, and used as the template for the second PCR reaction. The second semi-nested PCR product was a single clean band as shown in photo B. *Urma*-DRB alleles were confirmed by DNA sequencing.

```
                    HVR                                                                           HVR
                    10                                      20                                      30
DRB1*00101  TTG GAG GTG GCA AAG TCC GAG TGC TAT TTC ACC AAC GGG ACG GAG CGG GTG CGG TTC GTG GAA AGA TAC ATC CAT AAC CGG
Urma DRB01  G   AG  A   TAT     G           C                                          T C    CG      AG      T
Urma DRB02  G-- AG- A-- TAT --- G-- --- --- C--                                      --T C-- -CG --- AG- --- T-- --- ---
Urma DRB03  GT  AG  A   TAT     G           C                                                                 T
Urma DRB04  GT- AG- A-- TAT --- G-- --- --- C--                                      --T C-- -CG --- AG- --- T-- --- ---
Urma DRB05                      G           C                                                                 T
Urma DRB06  G   AG  A   TAT     G           C                                          T      CG      AG      T
Urma DRB07  G-- AG- A-- TAT --- G-- --- --- C--                                      --- --- --- --- --- --- T-- --- ---
Urma DRB08                      G           C                                          T C    CG      AG      T
Urma DRB09  G-- AG- A-- TAT --- G-- --- --- C--                                      --T C-- ACG --- AG- --- T-- --- ---
Urma DRB10  G   AG  A   TAT     G           C                                          T C    CG      AG      T
Urma DRB11                      G           C                                                                 T
Urma DRB12  --- --- --- --- --- G-- --- --- C--                                      --- --- --- --- --- --- T-- --- ---
Urma DRB13          A    T       T          C                                        C T C    TG      G       T
Urma DRB14  G-- AG- A-- TAT --- G-- --- --- C--                                      --T C-- -CG --- AG- --- T-- --- ---
Urma DRB15                      G           C                                          T C    CG      AG      T
Urma DRB16          A    T       T          C                                        C T C    TG      G       T
Urma DRB17  --- --- --- --- --- G-- --- --- C--                                      C-T C-- -TG --- G-- --- T-- --- ---
Urma DRB18                      G           C                                                                 T
Urma DRB19  --- --- A-- -T- --- -T- --- --- C--                                      --- --- --- --- --- --- T-- --- ---
Urma DRB20                      G           C                                         AT C    CG      G       T
Urma DRB21  G   AG  A   TAT     G           C                                          T C    CG      AG      T
Urma DRB22  --- --- --- --- --- G-- --- --- C--                                      --- --- --- --- --- --- T-- --- ---
Urma DRB23  G   AG  A   TAT     G           C                                          T C    CG      AG      T
Urma DRB24  --- --- A-- -T- --- -T- --- --- C--                                      C-T C-- -TG --- G-- --- T-- --- ---
Urma DRB25          A    T       T          C                                         AT C    CG      G       T
```

Fig. 4.2 Alignment of nucleotide sequences of 25 *Urma*-DRB alleles found in this study. A dash (-) indicates identity with the top sequence (DLA-DRB1*00101). Numbers indicate the nucleotide positions in the sequences. Three highly variable regions (HVR) were indicated.

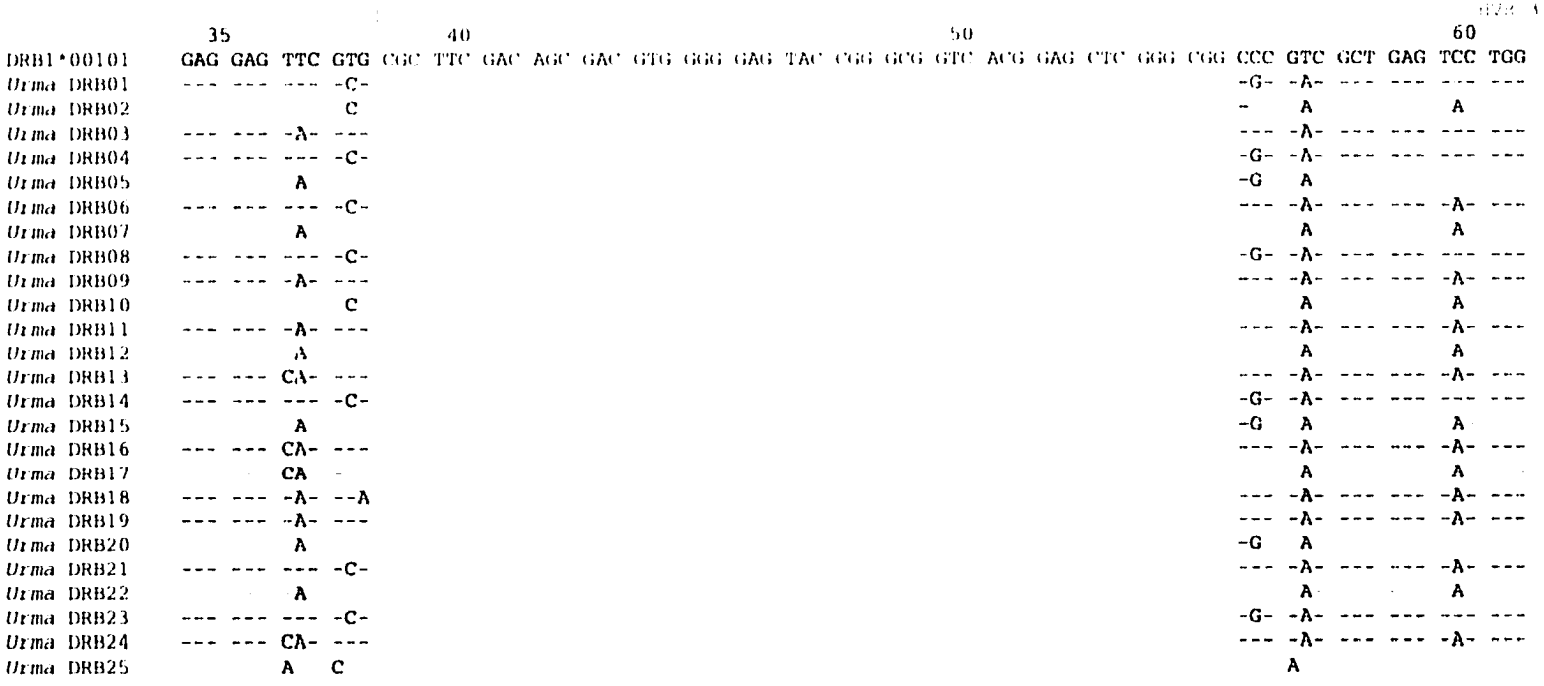


Fig. 4.2 (continued)

	65							70				
DRB1*00101	AAC	GGG	CAG	AAG	GAG	ATC	TTG	GAG	CAG	GAG	CGG	GCA	ACG
Urma DRB01	---	CC-	---	---	---	C--	---	---	-G-	-C-	---	--C	G--
Urma DRB02		CC				C			G	C		C	G
Urma DRB03	---	C--	---	---	---	C--	---	---	-G-	AG-	---	--C	GA-
Urma DRB04		C				C			G	AG		C	GA
Urma DRB05		C				C			G	AG		C	GA
Urma DRB06	---	CC-	---	---	---	C--	---	---	-G-	-C-	---	--C	G--
Urma DRB07		CC				C			G	C		C	G
Urma DRB08	---	C--	---	---	---	C--	---	---	-G-	-C-	---	--C	G--
Urma DRB09		CC				C			G	C		C	G
Urma DRB10						C				AG		C	GA
Urma DRB11	---	CC-	---	---	---	C--	---	---	-G-	-C-	---	--C	G--
Urma DRB12						C				AG		C	GA
Urma DRB13	---	CC-	---	---	---	C--	---	---	-G-	-C-	---	--C	G--
Urma DRB14		C				C			G	C		C	G
Urma DRB15		CC				C			G	C		C C	G
Urma DRB16	---	CC-	---	---	---	C--	---	---	-G-	-C-	---	--C	G--
Urma DRB17						C				AG		C	GA
Urma DRB18	---	CC-	---	---	---	C--	---	---	-G-	AG-	---	--C	GA-
Urma DRB19		CC				C			G	C		C	G
Urma DRB20		C				C			G	AG		C	GA
Urma DRB21	---	C--	---	---	---	C--	---	---	-G-	AG-	---	--C	GA-
Urma DRB22		CC				C			G	C		C	G
Urma DRB23	---	C--	---	---	---	C--	---	---	-G-	AG-	---	--C	GA-
Urma DRB24						C				AG		C	GA
Urma DRB25		C								AG		C	G

Fig. 4.2 (continued)

```
                                80
GTG GAC ACC TAC TGC AGA CAC AAC TAC GGG GTG ATT
                                                GGC



                                                GGC

                                                GGC
                                                GGC
                                                GGC
                                          A     GGC
          G GTG                                 GGC

          G GTG

                                                GGC
                                                GGC
                                                GGC
                                                GGC

                                                GGC

                                                GGC
                                                GGC

          G GTG
```

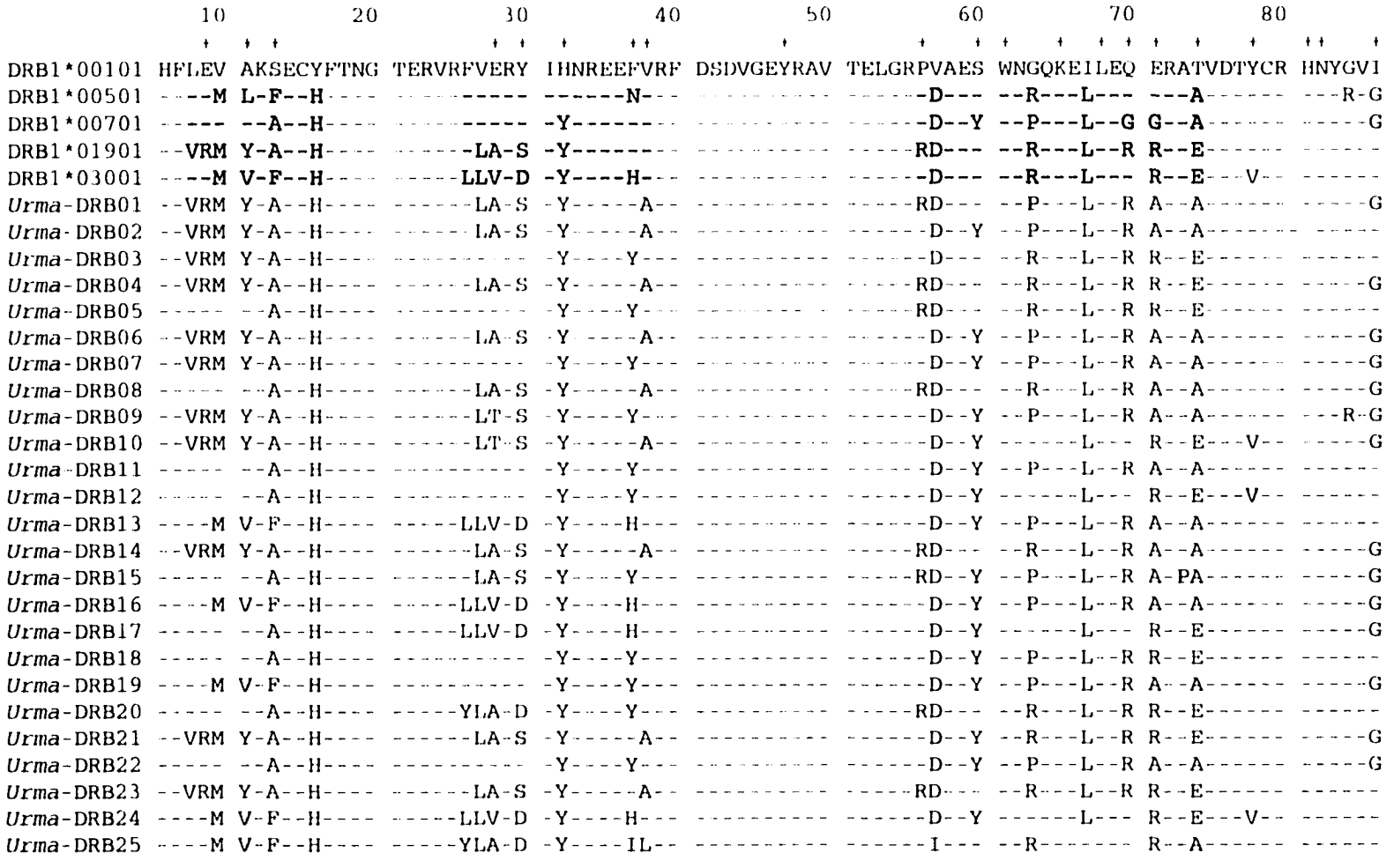


Fig. 4.3 Alignment of the deduced amino acid sequences of 25 *Urma*-DRB alleles. A dash (-) indicates identity with the top sequence (DLA-DRB1*00101). A cross (+) on the top of amino acid residue marks the position involved in the putative peptide binding site (Brown et al. 1993). Shaded areas in the DLA-DRB1 alleles indicate the three highly variable regions.

Fig. 4.4 Neighbor-joining phylogenetic tree of polar bear DRB (*Urma*-DRB) and canine DRB (DDRB1) exon 2 sequences. The tree was rooted with a dog DQB sequence, DQB2 (GenBank accession number: AF043147). Canine DRB allelic sequences were provided by Kennedy (Kennedy et al. 2001).

Table 4.1 Estimated rate of nonsynonymous (dN) and synonymous (dS) substitution and their ratio, where N is the number of codons in the DRB exon 2 tested and P is the probability of significance between dN and dS

Positions	N	dN	dS	dN/dS	P
Peptide binding	16	0.1717 ± 0.0409	0.0387 ± 0.0147	4.44	<0.01
Non-peptide binding	65	0.0561 ± 0.0109	0.0322 ± 0.0148	1.74	<0.02
All	81	0.0849 ± 0.0115	0.0387 ± 0.0147	2.19	<0.05

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Chapter V

MHC class II DRB and DQB polymorphism in a Doberman pinscher dog lineage[4]

Abstract

Objective: In order to investigate the impact of genetic bottlenecks on the genetic variability of the DRB and DQB genes at MHC class II loci, a pure Doberman pinscher lineage was analyzed.

Methods: MHC alleles were detected by PCR amplification of exon 2, cloning, and DNA sequencing.

Results: Only two DRB alleles, DRB1*00601 and DQB1*01201, were detected from 133 samples, while nine DQB alleles including four new ones were found from 51 samples. Sequence comparisons combined with a phylogenetic analysis showed that the four new DQB alleles likely result from different recombinations between the two most frequent alleles, DQB1*00901 (76%) and DQB1*00201 (55%). In addition, all samples tested for DQB showed heterozygosity, and three to four different DQB alleles were detected in some individuals, indicating the existence of two DQB loci in the dog.

Conclusion: The overall MHC allelic pattern in Doberman pinschers is very similar to that in musk ox, a wild species that experienced population crashes, implying the biased gene usage at the DQB locus among genetically bottlenecked populations.

Key words: MHC, DRB, DQB, genetic bottleneck, Doberman pinschers, polymorphism

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[4]Prepared for submission to Immunogenetics.

Introduction:

The dog has been used as an animal model in bone marrow and tissue transplantation for decades (Storb and Deeg 1985). The dog is also a valuable model for many human disease studies such as bone marrow transplantation and autoimmune diseases. Because both transplantation and many diseases have genetic links with the major histocompatibility complex (MHC) class II genes, the dog MHC region, termed dog leukocyte antigen or DLA, has been extensively investigated (Sarmiento et al. 1990 and 1993, Burnett et al. 1994, Wagner et al. 1996a, 1996b, and 1998, Wagner et al. 1999).

Like other mammalian MHC regions, DLA consists of three classes of genes: class I, class II, and class III. DLA class II molecules are heterodimeric glycoproteins that are expressed on the surface of the professional antigen presenting cells (APCs) such as dendritic cells, B cells, and macrophages, as well as on lymphocytes (Doxiadis et al. 1989). In mammals, the three major loci, DP, DQ, and DR, are located within the class II region. Each locus has at least one alpha chain and one beta chain, and each chain contains two domains. The highly polymorphic first beta domain contains a cleft which binds different antigen peptides and presents them to $CD4^+$ T cells to initiate the immune responses. This process eventually can results in elimination of infected parasites through endocytosis or rejection of transplanted bone marrow or tissues.

The objective of this study is to characterize MHC class II alleles at DRB and DQB loci in a pure breed dog lineage of Doberman pinschers, and to compare the number and diversity of these alleles with that of the wild musk ox. Since musk oxen have

experienced genetic bottlenecks and showed monomorphism at the DRB locus but relatively high polymorphism at the DQB locus (Chapter II), this comparison provided us with a interesting opportunity to investigate the evolution of MHC genes, especially the role of gene conversion in the generation of new MHC alleles.

Materials and Methods:

Animals:

One hundred and thirty-three Doberman pinscher blood samples were included in this study. Samples were collected from University of Florida and University of California at Los Angeles.

PCR, cloning and sequencing:

Genomic DNA was extracted from 200 µl of blood sample using a QIAamp blood kit (Qiagen, Chatsworth, California) according to the manufacture's instructions. Primers were synthesized based on the dog DLA-DRBB1 nucleotide sequence and the DLA-DQB nucleotide sequence, respectively (Kennedy et al. 1998, Wagner et al. 1996a & 1998). The PCR amplification of the DRB and DQB exon 2 region was performed with 5 µl of genomic DNA (200-400 ng) as template in a 50 µl reaction containing 5 µl of 10 × buffer, 5 µl of magnesium chloride (12.5 mM), 2 µl of 4 × dNTPs (2.5 mM), 1 µl of each primer (10 pmol), and 0.5 µl of *Taq* Polymerase (2 units). The PCR program consisted of an initial 3 min at 95°C, then total 35 cycles of 95°C for 30 s followed by 58°C for 30 s and

72°C for 1 min, and a final 10 min extension at 72°C. All PCR reactions were performed in 96-well plates using a GeneAmp PCR System 9700 (PE/Applied Biosystems). PCR products were monitored by running a 5 µl sample on a 2% agarose gel stained with ethidium bromide, then were purified either by Qiaquick PCR purification columns for direct sequencing or by a gel extraction kit (Qiagen) for cloning. The gel purified PCR products were cloned into pT7Blue-3 using Perfectly Blunt Cloning Kits (Novagen, Madison, Wisconsin). White colonies were picked up from agar plates and the plasmid DNA was purified using Wizard Plus Minipreps (Promega, Madison, Wisconsin). The plasmid DNA with the correct size of insert was chosen for DNA sequencing. An average of 5 to 10 clones were selected and sequenced for each sample. DNA sequencing on both strands was carried out on an ABI 373A instrument using fluorescent dye terminators (ABI, Foster City, California). All sequences were obtained from at least two different individuals or two separate PCR reactions.

DLA allele identification:

MatchTools (ABI, Foster City, California) was used for DLA allele typing. DLA-DRB and DLA-DQB allele libraries were generated by Lorna Kennedy, Chairman of the DLA nomenclature committee (Kennedy et al. 1999 and 2001).

Phylogenetic analysis:

Phylogenetic trees were constructed using the computer program PAUP 4.0 (Swofford 2000) by applying genetic distances estimated with Kimura's two-parameter

model (Kimura 1980). Bootstrap significance values greater than 50 for each branch were obtained based on 500 replicates.

Results:

For the DRB locus, only two alleles, DRB1*00601 and DRB1*01201, were detected. In a total of 133 samples examined, 89 samples (67%) showed homozygosity with either DRB1*00601 (n=72, 54%) or DRB1*01201 (n=17, 13%), while the remaining 44 samples (33%) showed heterozygosity with the combination of DRB1*00601 and DRB1*01201 (Fig. 5.1).

For the DQB locus, a total of nine alleles were found from the 51 samples tested (Fig. 5.2 and Fig. 5.3). All samples showed heterozygosity. The most frequent alleles were DQB1*00901 and DQB1*00201, each showed a frequency of 76% and 55%, respectively (Fig. 5.4). Other alleles, such as DQB1*00101, DQB1*00701, and DQB1*01301, were also detected with very low frequency (Fig. 5.4). In addition, four new DQB alleles were also detected and compared with the others (Fig. 5.2 and Fig. 5.3). Some samples were detected with three to four different DQB alleles (Table 5.1), indicating that there are two functional DQB loci in the dog MHC.

Seven dog DRB and DQB haplotypes have been detected in this study (Table 5.1).

Fig. 5.5 shows the phylogenetic analysis of the 23 published DQB alleles and the four new DQB alleles found in this study. There were four major DQB allele lineages that were strongly supported by bootstrap confidence level. The biggest allelic lineage with

many alleles including the four new ones clustered with the bootstrap value of 99. Seven out of nine DQB alleles found in this study clustered in lineage 1, while the other two, DQB1*00701 and DQB1*00101, were located in lineage 3 and 4, respectively.

Discussion:

This study has examined the MHC diversity at DRB and DQB loci in a pure breed dog lineage of Doberman pinschers. Previously published data showed that there are 39 DLA-DRB alleles and 23 DLA-DQB alleles detected from many different dog breeds (Kennedy et al. 1999 and 2001, Wagner et al. 1996a and 1998). The data from this study showed that only two DRB alleles and 9 DQB alleles including four new ones were detected from 133 samples of Doberman pinschers (Fig. 5.1, Fig. 5.2, and Fig. 5.3).

The pattern of dog MHC class II alleles at DRB and DQB loci revealed in this study is very similar to that of musk ox alleles (Chapter II), low genetic diversity at DRB but reletively higher genetic diversity at DQB. Musk ox are well known for having undergone genetic bottlenecks (Groves 1997), which may have greatly reduced allelic diversity at MHC loci. This explanation is correlated with the DRB locus since only one allele has been detected so far (Chapter II and Mikko & Andersson 1999). However, the explanation is not well correlated with the DQB locus even given the fact that there might be two DQB loci and nine different alleles have been detected (Chapter II). An alternative explanation could be that the DQB locus has been under greater selective pressure than the DRB locus, and thus more DQB alleles have been maintained to fight parasites.

Doberman pinschers are a pure dog lineage that is maintained by strict breeding. This dog lineage could be looked upon as man-made genetic bottleneck. The greater diversity of DQB alleles relative to DRB alleles in Doberman pinschers suggested that the DQB locus in Doberman pinschers might be under stronger selective pressure than the DRB locus. Preference of specific gene usage in different MHC class II loci has also been documented in many different species. For example, most mammalian species, such as humans and cattle (Parham & Ohta 1996, Mikko & Andersson1995), use DR predominately, while others use DQ primarily, such as mouse and musk ox (Mathis et al. 1983 and Chapter II). The extreme case could be mole rats that use DP exclusively (Schopfer et al. 1987).

There are three major mechanisms that have been proposed for the explanation of DNA motifs (short distinctive stretches of nucleotide sequences) shared between MHC alleles. The first mechanism is inheritance from ancestral sequences, the second one is convergent evolution (Klein & O'hUigin 1995), and the third one is gene conversion-like events (homologous recombination) (She et al. 1991, Gyllensten et al. 1991, and Parham & Ohta 1996). Considering the motif similarities among 7 out of 9 DQB alleles in this study (Fig. 5.2 & 3 and Table 5.2), it seems unlikely that the first and second mechanisms have been involved in the generation of new alleles by the sharing of motifs between alleles. If the four new DQB alleles are inherited from ancestral sequences, they should also appear in other dog breeds. To date, the published 23 dog DQB alleles are obtained from a relatively larger sample size and different breeds (Wagner et al. 1998, Polvi et al. 1997 & 1998), so the new DQB alleles seem to be Doberman pinscher-specific. In

addition, the frequencies of these new alleles are very low as compared with the two dominant alleles, DQB1*00901 and DQB1*00201 (Fig. 5.4), both are located in group 1 in Fig. 5.5. Furthermore, based on the phylogenetic analysis, all four new alleles plus DQB1*001301 are located in group 1 with the two dominant alleles, while the other two alleles, DQB1*00701 and DQB1*00101, are located in other groups (Fig. 5.5). Taken together, the first mechanism seems unlikely in this case. The second mechanism also seems to be not likely because there are 4 to 11 nucleotide differences between these 7 alleles and 8 of them are two nucleotides together forming 4 pairs (Fig.2). Convergent evolution is based on point mutation, and it could take several million of years to accumulate 4 to 11 nucleotide substitutions (Klein et al. 1993). If so, other dog breeds should also contain these alleles.

Gene conversion-like events seem to be the most likely mechanism to explain the generation of new DQB alleles in this study, since the four new alleles plus DQB1*01301 appeared to result from different recombination events between DQB1*00901 and DQB1*00201 (Table 5.2). Gongora et al. (1997) indicated in the investigation of HLA-DRB9 that an unknown mechanism may be involved in the generation of new alleles by encouraging gene conversion in some loci. The result from Gongora et al. (1997) is consistent with the above explanation for dog DQB alleles. Recently, Sale et al. (2001) reported in their investigation of chicken cell lines how gene conversion, the original mechanism for antibody diversification in chicken, was shifted to somatic hypermutation, a mechanism used mainly in some other species such as humans and mice. This research implied that some genes are involved in the control of gene conversion. To further

investigate the gene conversion mechanism in the generation of new MHC alleles, other model animals, such as mouse and cat, could be considered. Large sample size and pure lineage samples are crucial. Large sample size allows it possible to conduct horizontal experiments rather than conventional vertical ones, which saves a lot of time. Pure lineage samples allow horizontal experiments possible and also make it easier to detect the gene convention events.

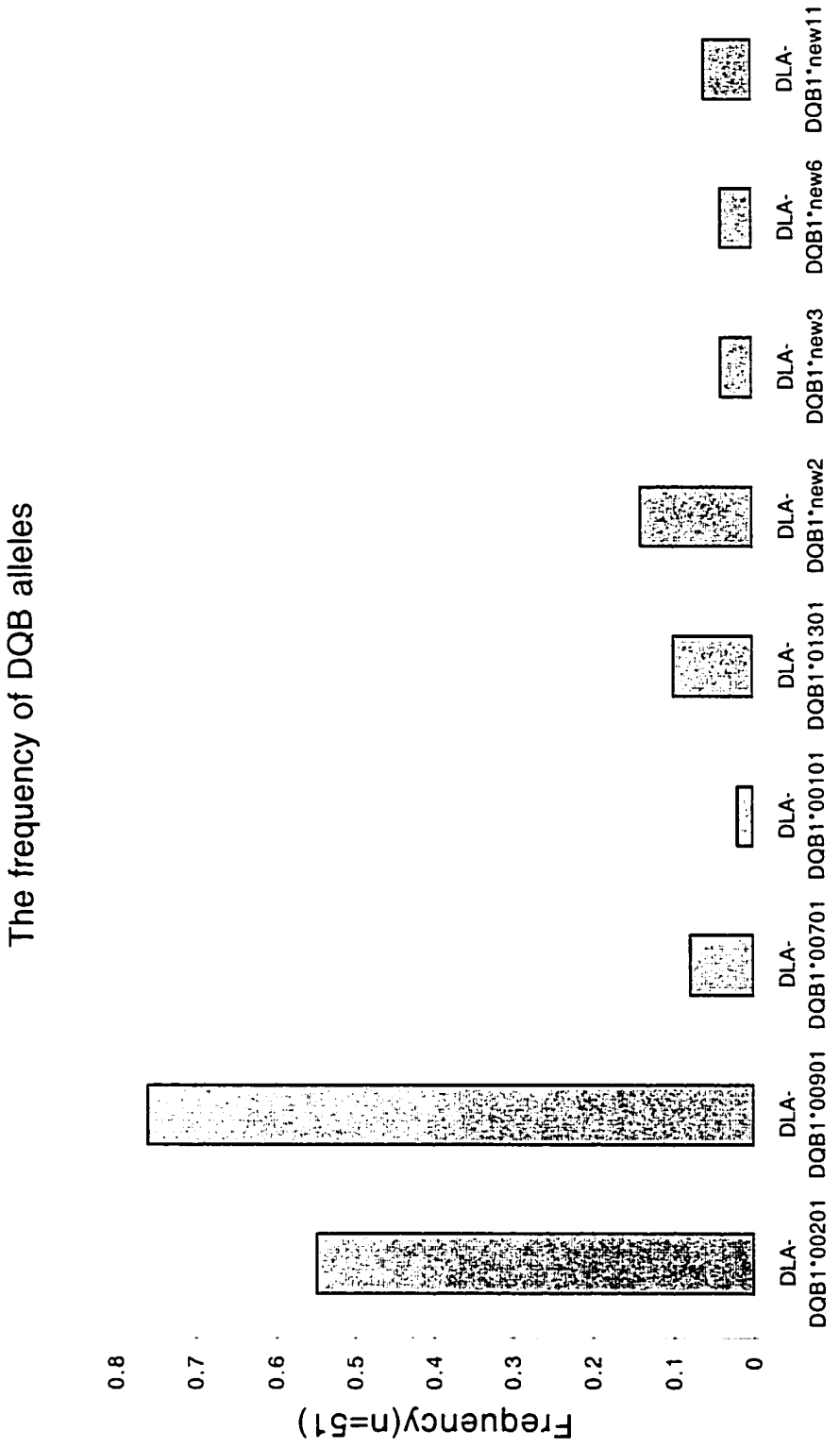

Fig. 5.1 Frequency of DLA-DRB alleles.

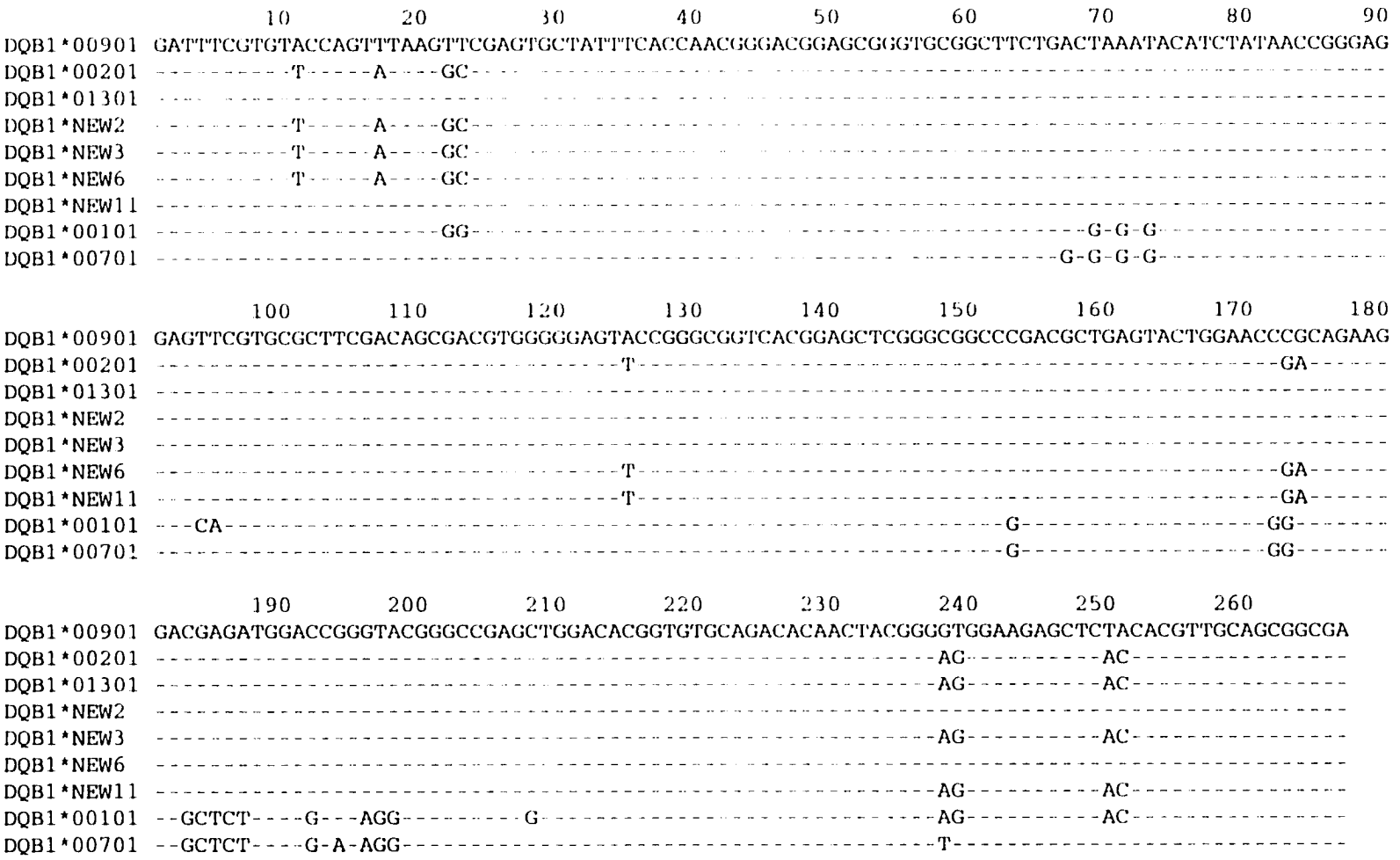

Fig. 5.2 Alignment of DLA-DQB alleles. A dash (-) indicates identity with the top sequence (DQB1*00901). Numbers on the top of the sequences indicate the nucleotide positions.

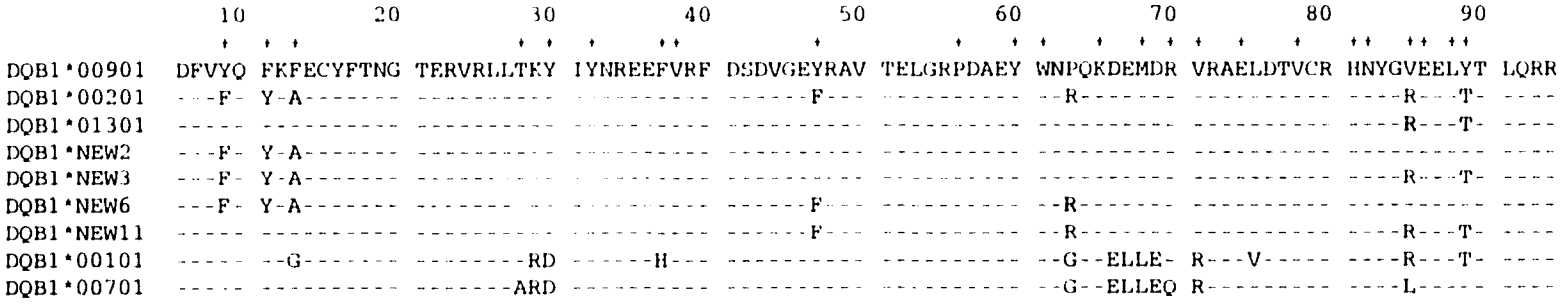

Fig. 5.3 Alignment of DLA-DQB amino acid sequences encoded by exon 2. A dash (-) indicates identity with the top sequence (DQB1*00901). The "+" symbol indicates the putative residues in the peptide binding site (Brown et al. 1993).

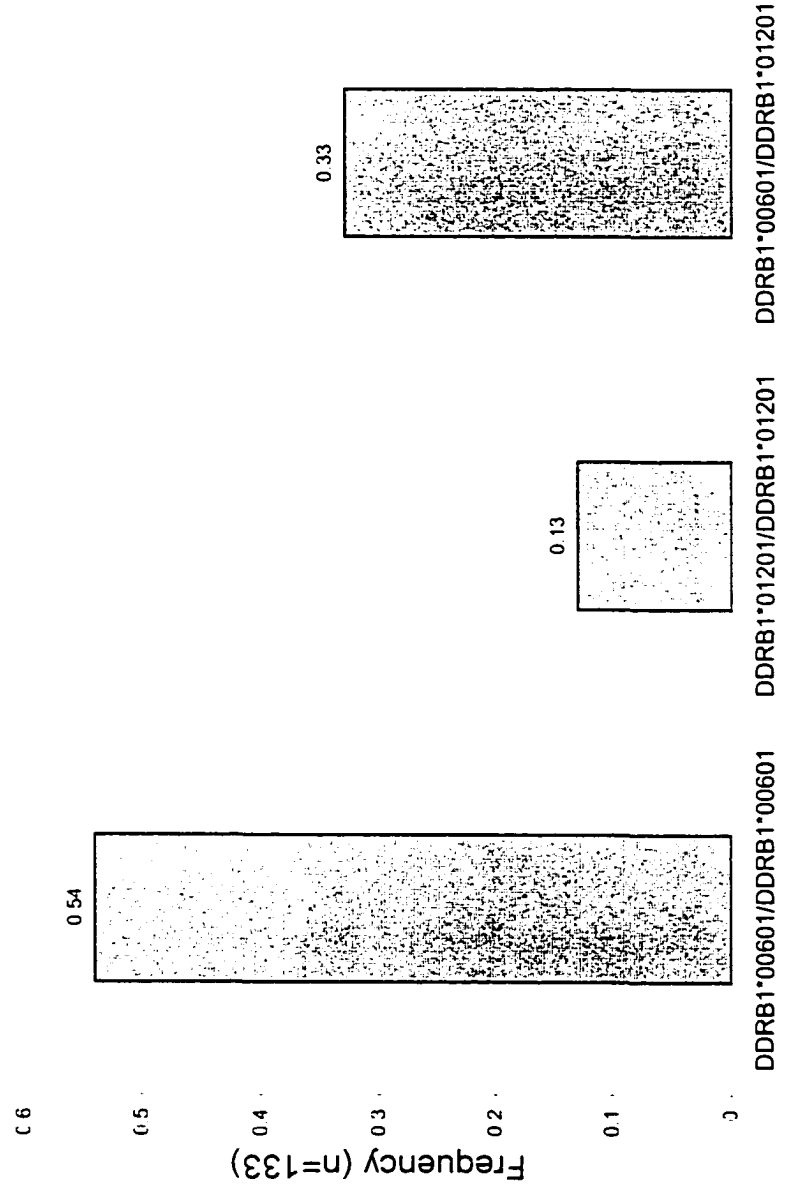

Fig. 5.4 Frequency of DLA-DQB alleles.

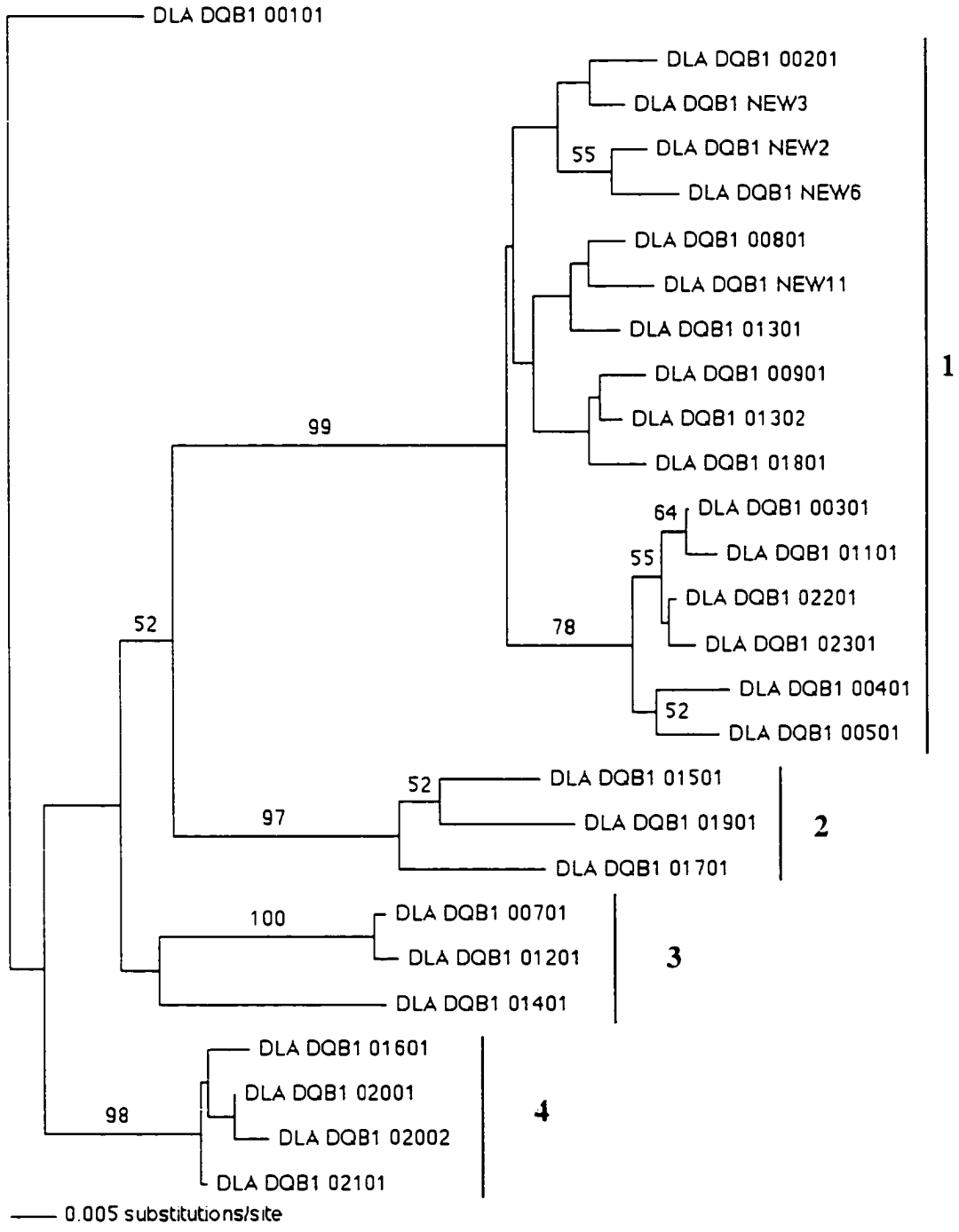

Fig. 5.5 Distance phylogenetic tree of the DLA-DQB exon 2 sequences. Numbers on branches indicate bootstrap values (500 replicates). Four major allele lineages with strong bootstrap support are indicated.

Table 5.1 Haplotypes of DLA-DRB and DLA-DQB with three or four alleles

Haplotype	DLA-DRB	DLA-DQB
1	DDRB1*00601/DDRB1*00601	DDQB1*00201 DDQB1*00901 DDQB1*new2
2	DDRB1*01201/DDRB1*01201	DDQB1*00201 DDQB1*00901 DDQB1*new11
3	DDRB1*00601/DDRB1*01201	DDQB1*00201 DDQB1*00901 DDQB1*01301 DDQB1*new3
4	DDRB1*00601/DDRB1*01201	DDQB1*00201 DDQB1*00901 DDQB1*NEW6
5	DDRB1*00601/DDRB1*00601	DDQB1*00201 DDQB1*00901 DDQB1*01301
6	DDRB1*00601/DDRB1*01201	DDQB1*00701 DDQB1*00901 DDQB1*NEW3
7	DDRB1*00601/DDRB1*01201	DDQB1*00901 DDQB1*NEW2 DDQB1*NEW11

Table 5.2 DLA-DQB sequences showing the positions of different polymorphic nucleotides. A dash (-) indicates identity to the first sequence. Numbers of nucleotide position are based on Fig. 2

	Position										
					1	1	1	2	2	2	2
	1	1	2	2	2	7	7	3	3	5	5
Sequence	1	7	2	3	5	3	4	8	9	0	1
DQB1*00901	A	T	T	T	A	C	G	G	T	T	A
DQB1*00201	T	A	G	C	T	G	A	A	G	A	C
DQB1*01301	-	-	-	-	-	-	-	A	G	A	C
DQB1*new2	T	A	G	C	-	-	-	-	-	-	-
DQB1*new3	T	A	G	C	-	-	-	A	G	A	C
DQB1*new6	T	A	G	C	T	G	A	-	-	-	-
DQB1*new11	-	-	-	-	T	G	A	-	-	-	-

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Chapter VI

Concluding remarks and future perspectives

MHC genetic diversity characterized mainly at class II DR and DQ loci in some northern species was described in the previous chapters. The aim was to conduct a comparative analysis of genetic diversity at MHC class II loci in these northern species in an attempt to evaluate the MHC genetic diversity and evolution in the Arctic. Specific goals met in this thesis are: 1. Assessment of the MHC locus usage preference between DRB and DQB in musk ox (*Ovibos moschatus*) and moose (*Alces alces*) (Chapter II); 2. Characterization of the level of MHC DRB variation in reindeer and caribou, *Rangifer tarandus*, a species found in gregarious populations as compared to solitary musk ox and moose (Chapter III); 3. Assessment of the level of MHC DRB variation in polar bears (*Ursus maritimus*), the first such attempt in bears and the only carnivores included in the comparative study in this thesis (Chapter IV); 4. Evaluation of the impacts of genetic bottlenecks on MHC genetic diversity in a pure Doberman pinscher dog lineage (*Canis familiaris*) (Chapter V), and comparion to result obtained from musk ox (*O. moschatus*), a natural population that has experienced genetic bottlenecks.

This chapter contains concluding remarks and future perspectives. Based on the work conducted in the previous chapters, suggestions for future investigations required to fully understand the significance of MHC genetic diversity and its evolution in the Arctic are discussed.

MHC polymorphism and expression:

Because of the unique geographical features in North America, northern species have been influenced dramatically by repeated climatic oscillations and range changes during the recent ice ages. A population may pass through many rounds of such expansion and contraction, rapid colonization and adaptation to new environments and new neighbors. The post-glacial population expansions are most likely the reason for the reduced genetic diversity detected in many northern species (Hewitt 2000). The current genetic structure in northern species is the direct result of the interplay among biology, geography, and climatic shifts.

Although high levels of genetic diversity at the MHC have been detected in most mammalian species (Swarbrick et al. 1995, Yuhki and O'Brien 1997), there are several cases that demonstrate low levels of MHC genetic variability (O'Brien et al. 1985, Slade 1992, Ellegren et al. 1993, Mikko et al. 1995). The level of MHC genetic variability differs considerably not only at the species level, but also between MHC loci, for instance, the monomorphic DRB and relative polymorphic DQB in musk ox (Chapter II). Many factors have been discussed for the generation of MHC variability. These factors include speciation events, selective pressure, genetic drift, and population bottlenecks (Parham and Ohta 1996). To better understand the evolutionary significance of MHC polymorphism in the Arctic, more comparative investigations of northern species are needed.

While low MHC variation is associated with the history of the individual species, it could also be explained by the lack of appropriate PCR primers specific for that species. MHC genetic diversity is characterized in most studies by PCR-based methods, with primers derived from closely related species, the PCR amplification of MHC genes may not represent all MHC alleles in that species because of sequence differences in the primer region. This scenario happened in the detection of moose DQB alleles described in Chapter II. PCR primers used for the detection of moose DQB alleles were adopted from cattle. When moose DQB cDNA was amplified and sequenced using one reverse primer locating the exon 3 region, the cDNA sequence in the first reverse primer region was found to be quite different from the cattle primer used, indicating a new allelic lineage amplified unsuccessfully using the cattle reverse primer. New reverse primer was then synthesized according to the moose cDNA sequence and used to amplify the second moose DQB lineage.

Several approaches could help to solve the PCR technique limitation. One approach is to compare the MHC sequences of closely related species before adopting the PCR primers for the target species. This method, which has been used in investigations for this thesis, can avoid primer bias and cover as many MHC alleles as possible. The second approach is to characterize the expression of amplified MHC alleles or loci through reverse PCR (RT-PCR) or cDNA library screening. This method can help to evaluate which locus is functional and its importance in the immune system, and to discover unknown loci which may comprise additional MHC polymorphism. The third approach is to clone and sequence the MHC region of the target species by DNA library

screening such as Bacterial Artificial Clone (BAC) screening. This method can determine how many MHC loci are present overall in this species and how many of them are pseudogenes. PCR primers synthesized based on the sequence obtained from the above approaches will amplify specific MHC locus alleles and cover the majority of the MHC variation.

Identical MHC alleles have been found to be shared among different species. For the class I loci, one C locus allele is shared by two different chimpanzee species, another HLA-G-like allele is shared by two tamarin species (Cooper et al. 1998, Evan et al. 1998). For the class II loci, four DRB alleles are shared by polar bears and dogs, and another four DQB alleles are shared by musk ox and moose (Chapter II). These are dramatic examples of stringent trans-species polymorphism (Klein 1987). It is possible to find more such examples by conducting extensive comparative studies among related species such as snow-shoe hare and rabbit for example. The sharing of an identical MHC allele by two different species provides an opportunity to determine the evolutionary rates of MHC genes (Cooper et al. 1998). It also provides a unique opportunity to investigate the host-pathogen relationships among different species and in different ecological environments.

MHC polymorphism and parasite association:

The maintenance of high levels of genetic diversity observed at MHC in vertebrates is widely believed to be due to the parasite-driven balancing selection (Klein

and O'hUigin 1994). There are several examples which provide empirical evidence that strongly supports the hypothesis (Paterson et al. 1998, Carrington et al. 1999, Jeffery et al. 1999). The sharing of identical MHC alleles between different species opens a unique way for direct comparison of the spectrum of parasites in these species. In vitro peptide binding assays using the molecule of the identical MHC allele may help to determine the spectrum of peptides that the molecule can bind (Raddrizzani et al. 1997, Sturniolo et al. 1999), and the combination of these peptides may help to determine the parasite associated with related species. Alternatively, the association of the identical allele with the involved parasite can be determined by screening the spectrum of parasites in both species, although most parasite information is currently incomplete.

The MHC class II allelic sharing between two distantly related species, musk ox and moose living in almost the same environment and polar bear and wolf living in overlapping but different distributions, provides such an opportunity to determine the common parasite antigens associated with these two species. Parasite infection has also been found in moose (Addison and McLaughlin 1988), but there seems to be no major common parasites reported between these two species (Eric Hobert, personal communication), making it difficult to determine which parasite is likely associated with the identical MHC DQB alleles shared by these two species. Future work may need to focus on the parasite association with these shared MHC alleles. The same is true for polar bears and dogs. Dog has been used as an animal model for decades. It is much easier to investigate the MHC allelic association with parasite antigens using dog rather than polar bear. Overall, the determination of MHC allelic association with parasite

antigens for a shared identical MHC allele between two different species would provide a clue for the evolution of the allele as to whether or not it has been under strong selection.

Recombination in the generation of new MHC alleles:

It is still an open question whether or not recombination mechanism is involved in the generation of new MHC alleles. Supporting lines of evidence insist that the reshuffling of allelic motifs has been found in numerous investigations with a wide range of different species (Walkins et al. 1992, Yuhki and O'Brien 1994, Swarbrick et al. 1995). Opposing points are that these similar or identical allelic motifs can be the result of convergent evolution (Klein and O'hUigin 1995). Recombination-like events were observed in musk ox and a Doberman pinscher dog lineage at the MHC DQB locus (Chapter II and Chapter V). Interestingly, both of these populations have experienced genetic bottlenecks which could reduce the general genetic diversity. Greater abundance of MHC alleles at the DQB locus than that of the DRB locus provides evidence of the importance of DQB alleles in the bottlenecked populations. This may be due to the broader binding capability of DQB alleles (Raddrizzani et al. 1997), and thus provide more efficient immune surveillance for the genetic bottlenecked hosts that have less MHC alleles at other loci. To confirm the role of recombination in the generation of new MHC alleles, model animals that have short reproductive cycle, fewer MHC alleles, and are easy to manipulate would be advantageous. Fish, such as sticklebacks (*Gasterosteus aculeatus*) (Reusch et al. 2001), seem to be a good candidate for the choice. Stickleback

as a species has been undergone intensive molecular and evolutionary investigations because of its importance and convenience. It would be easier to utilize the existing molecular information of MHC obtained from this species to design and execute the experiment on the role of recombination. Horizontal rather than vertical experiment would be possible by using sticklebacks. Other model animals such as the mouse and rat may also be considered.

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